Report on the External Peer Review Meeting of Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin, and Microcystins LR, RR, YR, and LA

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Notice

This report was prepared by Eastern Research Group, Inc. (ERG), an EPA contractor, as a general record of discussion during the External Peer Review Meeting of Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin, and Microcystins LR, RR, YR, and LA, held January 10, 2007, in Cincinnati, Ohio. This report captures the main points and highlights of the meeting. It is not a complete record of all details discussed, nor does it embellish, interpret, or enlarge upon matters that were incomplete or unclear. Statements represent the individual views of meeting participants.

Contents

1. Introduction

The 1996 Safe Drinking Water Act (SDWA) Amendment requires that the U.S. Environmental Protection Agency (EPA) make regulatory decisions on at least five contaminants from the Contaminant Candidate List (CCL) every 5 years. The 1998 CCL (CCL 1) and 2005 CCL (CCL 2) include cyanobacteria (blue-green algae), other freshwater algae, and their toxins as microbial contaminant candidates.

EPA's National Center for Environmental Assessment (NCEA) developed Toxicological Reviews for several cyanobacterial toxins—anatoxin-a, cylindrospermopsin, and microcystins LR, RR, YR, and LA—to support the health assessment of unregulated contaminants on CCLs 1 and 2. These documents compile and evaluate the available data regarding toxicity, human health effects, animal exposure studies, mode of toxin action, susceptible population groups, and clinical and epidemiological studies of these selected cyanobacterial toxins to aid EPA's Office of Water in regulatory decision-making and/or the development of health-based exposure guidelines.

The draft Toxicological Reviews for the cyanobacterial toxins anatoxin-a, cylindrospermopsin, and microcystins have undergone internal EPA review for scientific accuracy and compliance with EPA risk assessment guidelines and procedures. On January 10, 2007, Eastern Research Group, Inc. (ERG), an EPA contractor, convened an independent panel of six nationally and internationally recognized experts (Appendix A) in a peer review meeting to further evaluate the accuracy of the content and interpretation of the findings presented in these documents. In the Charge to Panelists (Appendix B), EPA asked the panelists to consider general questions regarding document format and content, as well as document-specific questions regarding the derivation of reference values.

Appendix C provides a list of those who attended the meeting as observers. A phone line was available for any observers who wished to attend remotely. The meeting agenda (Appendix D) included an opportunity for observer comment.

This report summarizes presentations and discussions that took place during the peer review meeting. For organizational efficiency, this report groups discussions by topic, rather than strictly chronologically.

2. Opening Remarks

Jan Connery (ERG) opened the workshop by welcoming the expert review panel members and observers (Appendices A and C). She reviewed the meeting agenda (Appendix D) and asked the panelists to introduce themselves.

Connery then introduced Belinda Hawkins, the cyanotoxins chemical manager, from EPA's National Center of Environmental Assessment. Hawkins briefly provided background information regarding the documents under review (Appendix E).

Under the Safe Drinking Water Act (SDWA), EPA develops the CCL, which includes unregulated substances that are or may be present in drinking water supplies and may require regulation under the SDWA. In 2001, EPA, researchers, and experts met to develop a list of target algal contaminants likely to pose a health risk in drinking water. This group identified anatoxin-a, cylindrospermopsin, and microcystins as the cyanotoxins of highest priority based on their potential health effects, occurrence in the United States, susceptibility to drinking water treatment techniques, and stability.

As a result, EPA prepared toxicological reviews to compile toxicity data and to evaluate these data for anatoxin-a, cylindrospermopsin, and microcystins (LR, RR, YR, and LA). The toxicological reviews are intended to support the health assessment of these cyanotoxins, which are unregulated substances on the CCL. The documents are not intended to provide a comprehensive description of the chemical or toxicological nature of these cyanotoxins. They serve only as hazard assessments, not complete risk assessments. As such, the documents contain only data and evaluations relevant to hazard identification and dose-response assessment. Cyanotoxin occurrence and exposure descriptions and risk characterization discussions are excluded from the documents. Hawkins emphasized that the panelists should review the documents as hazard assessments and not as complete risk assessments.

In addition, EPA prepared the toxicological reviews in accordance with EPA hazard assessment methodologies, which can differ from other regulatory agencies' methodologies. EPA requires assessment of single chemical exposures (not mixtures), exclusion of uncharacterized cell extract studies, consideration of only relevant exposure routes for dose-response assessment, separation of non-cancer and cancer assessments, and use of five standard uncertainty factors. The five standard uncertainty factors address variation in human sensitivity, animal-to-human extrapolation, no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) extrapolations, subchronic-to-chronic extrapolation, and database uncertainty.

Hawkins stated that EPA will consider the panelists' comments and recommendations, prepare a response to these comments, and revise the documents accordingly. EPA will then release the revised documents for clearance. No additional public comment or peer review will occur. As such, Hawkins stressed the importance of this peer review.

3. Observer Comments

Connery opened the meeting to observer comments and two observers, participating via teleconference, provided comments.

Andy Reich, Florida Department of Health, noted that agencies in Florida have been struggling with assessing the public health impact of cyanotoxins for a number of years. He supported EPA's efforts to evaluate the toxicity of cyanotoxins and looked forward to the outcome of the peer review and ongoing EPA efforts.

Jan Landsberg, Florida Fish & Wildlife Conservation Commission, concurred with Andy Reich and also noted her interest in the outcome of EPA's efforts.

4. Panelist General Comments

The panel chair, Daniel Dietrich, began discussions by highlighting concepts that he felt the panelists should consider when conducting the review (Appendix F). These included data quality, mode of action/mechanism, magnitude of deficiencies in the assessment (e.g., moderate versus serious), and possible and feasible short- and long-term database improvements. He also noted that the panelists should carefully weigh the statistics presented in the toxicological data to ensure meaningful use of statistical analyses.

The panelists addressed each of the three toxicological reviews in sequence (anatoxin-a, cylindrospermopsin, and microcystins) beginning with a discussion of the toxin-specific charge questions followed by a discussion of the general charge questions (Appendix B). At the conclusion of document-specific discussions, the panelists developed a consensus statement summarizing conclusions and recommendations. During document-specific discussions, panelists also noted general comments that pertained to all three documents or the overall review process. These comments included the following:

- Panelists noted that they remained in agreement with the opinions expressed in their pre-meeting comments (Appendix G), unless otherwise noted during meeting discussions. They referred EPA to the pre-meeting comments for detailed, document-specific comments.

- One panelist noted that the differences between exposure and risk were not clearly identified in the documents.

- Several panelists noted that the documents, particularly the toxicological review for cylindrospermopsin, would benefit from a more expansive table of contents, especially for the appendices, to improve document navigation.

- The panelists recommended that EPA limit discussions of toxin sources to only those organisms proven to produce a toxin in culture. References to organisms in blooms or anecdotal reports of organisms as sources can be misleading.

- The panelists suggested that EPA distinguish between natural and synthetic toxins, as well as between pure and bloom material. Preference should be given to pure natural material. Bloom material should only be used as supporting data.

- Improved forensic capabilities are needed to rapidly and properly identify cyanotoxins associated with animal and human intoxication events.

5. Anatoxin-a

Consensus Statement

The panelists prepared the following consensus statement regarding the anatoxin-a toxicological review:

> **Acute Effects:** The initial report on the death of a Wisconsin teenager due to anatoxin-a has too many confounding factors to be included in the weight-of-evidence approach for deriving the reference value.
>
> **Additional Studies:** The current toxicity database for anatoxin-a is inadequate. However, all available data (conscious of confounding factors) are acceptable for a point of departure in defining further studies. Considering anatoxin-a's mechanism of action as a nicotinic agonist with a steep dose-response, experimentally well-designed acute oral studies should have the highest priority, followed by subchronic studies using unpublished EPA data (McPhail et al., 2007).[1] There is a definite need for mechanistic and kinetic studies. In addition, to reduce uncertainty, species other than rodents would be ideal. Based upon available toxicity data, cancer studies would have a lower priority than the above more-important studies.

Discussion of Charge Questions 1–5: Derivation of Reference Values

The panelists agreed that the data were inadequate to support the derivation of oral reference doses (RfDs) for acute and chronic exposure durations.

Panelists noted that deriving an acute RfD is important due to the high acute toxicity of anatoxin-a. Appropriate studies for deriving this value, however, are not available. The multiple acute studies presented in toxicological review for anatoxin-a were not appropriate for deriving an acute RfD because dose-response data were lacking or synthetic toxins were administered. Use of a synthetic material was considered a study weakness. In addition, existing studies mainly used intraperitoneal injection as the route of exposure. Extrapolating to the oral route of exposure is inappropriate.

In considering toxicity data available for an acute exposure duration, the panelists discussed the single case report of several Wisconsin teenagers exposed to anatoxin-a while swimming during an algal bloom (Behm, 2003; Carmichael et al., 2004). The panelists noted several deficiencies with this case report. Sampling results were negative for organisms known to produce anatoxin-a; however, sampling did not occur immediately following the exposures. One panelist noted that anatoxin-a is unstable in bloom situations. In addition, animal intoxication incidents have failed to detect anatoxin-a after exposure due to rapid breakdown of the toxin. This panelist was

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[1] Unpublished data cited as an additional study by the panelists; results presented at the 2006 Society of Toxicology convention (R. C. MacPhail, J. D. Farmer, and K. A. Jarema, Comparison of Nicotinic Antagonists in Blocking the Effect of Anatoxin-A and Nicotine on the Motor Activity of Rats, 1491, The Toxicologist — An Official Journal of the Society of Toxicology, Volume 90, Number S-1, March 2006).

concerned about definitively concluding that the effects presented in the case report were a result of anatoxin-a exposure alone. Overall, this incident highlights the need for improved forensic methods to assess algal blooms and to identify the involved organisms and toxins. This case report also highlights the lack of human acute exposure data, as well as the limited usefulness of human intoxication studies. Rather than delete the discussion of this case report from the document, as suggested by one panelist, the panelists agreed that EPA retain the case report in the toxicological review for anatoxin-a, but clearly indicate that the case report is not appropriate for risk assessment. Due to confusion regarding the events surrounding this incident, the case report discussion should document the activities that occurred, the analyses performed, the analyses timing as related to exposure and onset of illness, and the outcomes of these analyses. The discussion should also mention that no other cause of death/illness could be identified. The possibility of blue-green algal toxin exposure, although not confirmed due to inappropriate timing of analyses, could not be excluded. EPA should also reference and include within the toxicological review for anatoxin-a the coroner's report for this incident.

One panelist provided a summary of unpublished data from an acute study of mice orally dosed with anatoxin-a fumarate (MacPhail et al., 2007). This study examined the effects of anatoxin-a on motor activity and found a dose-related decrease in vertical activity in survivors at a no-observed-effect level (NOEL) of 12 milligrams/kilograms (mg/kg) and a lowest-observed-effect level (LOEL) of 18 mg/kg. Motor activity recovered 5 days after exposure. This study indicates that multiple endpoints appropriate for risk assessment may exist. Scientists, however, do not sufficiently understand anatoxin-a's mechanism of action to assess possible additional endpoints. A panelist suggested that these data may indicate that the mechanism for anatoxin-a toxicity is different from the mechanism for nicotine toxicity, which raises the possibility that anatoxin-a is not just an agonist of nicotinic receptors. Another panelist noted that the MacPhail et al. (2007) study evaluated exposures to a mixture; therefore, the link between anatoxin-a and change in motor activity was not certain. Panelists recognized that study limitations existed, including the small sample size (n=6) per dose group. Even if published, this study might not be useful for deriving a reference value because of these limitations.

The panelists agreed that the 28-day gavage study in mice (Fawell and James, 1994; Fawell et al., 1999) served as an appropriate principal study in deriving the short-term oral RfD. They discussed the use of 2.5 milligrams/kilograms-day (mg/kg-day) versus 0.1 mg/kg-day as the point of departure. Several panelists felt that the lower dose should serve as the point of departure because the authors could not conclusively determine that observed mortalities at doses below 2.5 mg/kg-day were unrelated to anatoxin-a exposure. A panelist noted that the findings from MacPhail et al. (2007) suggest that a slow-acting mechanism of action may be involved in anatoxin-a toxicity at low doses. Other panelists felt that 2.5 mg/kg-day served as an appropriate point of departure and that the mortalities observed at the lower dose were unrelated to anatoxin-a exposure. The mice died several hours after anatoxin-a administration. A panelist noted that anatoxin-a is a fast-acting substance, and although time to death can vary, mortality after several hours is not likely. One panelist speculated that these results open questions about potential anatoxin-a effects just below lethal dose concentrations. After discussion, the panelists agreed that 2.5 mg/kg-day is an appropriate point of departure, however, additional studies, using a larger sample size and a more rigorous statistical design, were needed to support this finding.

When evaluating the 28-day gavage study in mice, the panelists also reviewed concerns related to the statistical analyses of study data. One panelist noted that a small change in results (e.g., an increase of one death) could significantly impact the statistical analyses for a study with a small number of test subjects. Several panelists noted the small study size as a weakness in the 28-day gavage study. A panelist noted that additional studies should use larger study animal groups and focus on fewer, non-fatal, endpoints. This panelist noted that statistical analysis of small study groups might be incapable of identifying significant changes in endpoints more subtle than mortality.

Of all the studies used to derive reference values for the cyanotoxins under review, one panelist stated that the weakest was the 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980) selected as the basis for the subchronic oral RfD for anatoxin-a. The study examined two dose groups consisting of female rats and examined a small number of endpoints. In contrast to his pre-meeting comments, this panelist concluded that this 7-week study should not be used to derive a subchronic RfD. Another panelist agreed that the study was inappropriate for deriving the subchronic RfD because it identified only a free-standing NOAEL, which provides insufficient data for selecting an appropriate point of departure. The resulting RfD may be much lower than necessary to protect public health. This panelist expressed particular concern that once derived, the subchronic RfD would be inappropriately used to develop a chronic oral RfD. The remaining four panelists agreed that the 7-week drinking water study contained significant flaws, but felt the study could serve as the basis for a subchronic oral RfD. One felt that because so few studies are available, even deficient studies should be considered. EPA, however, should clearly state that additional data supporting the RfD are needed. Others agreed that additional supporting studies were necessary. One panelist noted that, had the study considered alternate endpoints, effects might have been seen at lower exposure doses.

As suggested by the panel prior to the meeting, one panel member provided a brief summary of an intranasal exposure study in mice (Fitzgeorge et al., 1994; Benson et al., 2005[2]). This panelist noted that the lethal dose (LD_{50}) for the intranasal exposure was similar to the 2.5 mg/kg-day dose used to derive the short-term oral RfD. One panelist noted that Benson et al. (2005) considers exposure to microcystin-LR, not anatoxin-a.

The panelists agreed that the uncertainty factors applied to the short-term and subchronic oral RfDs were appropriate.

Discussion of Question 1: Cancer Assessment

The panelists agreed that insufficient data are available to complete a cancer assessment for anatoxin-a. Based on existing data and personal experiences with anatoxin-a, the panelists also agreed that assessing the carcinogenic potential of the toxin should be a low priority.

[2] Cited as additional studies by the panelists; Appendix F

Discussion of General Charge Questions 1–3

Overall, the panelists felt that the anatoxin-a toxicological review was a well-written and thorough document.

One panelist noted concern about the organisms identified as producing anatoxin-a. The document references cultured organisms, bloom materials, and organisms from anecdotal evidence as potential sources of anatoxin-a. Panelists agreed that EPA should identify only organisms proven to produce anatoxin-a in culture as known sources. Including bloom materials or anecdotal information is misleading, especially for utilities that base water use decisions on detections of organisms, not toxins.

EPA completed the literature search for anatoxin-a in May 2006. Hawkins confirmed that the literature search consisted of an extensive review of publicly available materials and databases. The panelists strongly recommended that EPA update this literature. Hawkins noted that EPA planned to do so.

During discussions of the charge questions related to the derivation of anatoxin-a reference values, the panelists included references to additional existing studies that EPA should consider. The panelists also recommended additional research that would reduce uncertainties and improve future assessments of anatoxin-a. The panelists discussed conducting well-designed rodent studies with large study groups. Studies of small numbers of pigs, which better mimic human physiology, would provide supporting data to reduce uncertainties. The consensus statement for anatoxin-a, provided earlier in this section, summarizes and prioritizes recommendations for additional research.

One panelist noted that EPA should consider the findings, when released, of the interagency International Symposium on Cyanobacterial Harmful Algal Blooms held in September 2006.

6. Cylindrospermopsin

Consensus Statement

The expert panel prepared the following consensus statement regarding the cylindrospermopsin toxicological review:

> **Subchronic Effects:** Kidney effects in the male mouse (Humpage and Falconer, 2003) are most likely male-mouse specific. While this may be an appropriate point of departure, it needs to be interpreted properly. These sections should be revised in the document.
>
> **Chronic Effects:** Deficiencies in knowledge on the mode of action make it difficult to derive a chronic dose. Other mechanisms, such as cytotoxicity and genotoxicity, may occur at lower doses.
>
> **Carcinogenicity:** Although no conclusions can be currently drawn on the carcinogenicity of cylindrospermopsin, there is suggestive mechanistic evidence and a preliminary study

that point to carcinogenic potential (as cited in public comments submitted by Ian Falconer).

Additional Studies: There is a high need for well-designed studies with appropriate endpoints for acute and chronic toxicity and cancer assessment. An acute study should include more than 10 animals of both genders per dose group and examine 4 to 5 dosing groups.

Discussion of Charge Questions 1–6: Derivation of Reference Values

The panelists agreed that available oral toxicity information is inadequate for the derivation of an acute or short-term oral reference value. The toxicological review for cylindrospermopsin describes an outbreak in the Palm Island community in Queensland, Australia, in 1979 (Blyth, 1980; Griffiths and Saker, 2003). Cylindrospermopsin was speculated as a cause of this outbreak, with children being more susceptible to cylindrospermopsin effects than adults due the high number of children affected versus adults. The panelists discussed several possible causes for this discrepancy. One panelist noted that water experiencing a bloom or treated with copper sulfate smells and tastes bad. Adults may have avoided the water, whereas the children did not. Another noted the children are more susceptible simply because of their lower body weight. Children also may have been exposed while swimming, as well as drinking water. Children are also more susceptible to dehydrating illnesses (e.g., diarrhea and vomiting), which could lead to more hospitalizations for the children. At the time of the incident, copper sulfate, which was used to treat the algal bloom, was initially implicated as the possible cause of the outbreak. However, investigators concluded that the clinical picture and range of symptoms were not consistent with copper intoxication. The cyanobacterium (*Cylindrospermopsis raciborskii*) was isolated and cultured from the Palm Island reservoir and was shown to be toxic to mice, which exhibited symptoms similar to those found in the affected population. The alkaloid cylindrospermopsin, isolated from cultures of *Cylindrospermopsis*, was later identified as the principal cyanotoxin present that exhibited toxicity similar to symptoms described in the outbreak. An association between cylindrospermopsin and symptoms was present, but a direct dose-response relationship could not be established.

Although the 11-week gavage study in mice (Humpage and Falconer, 2003) could serve as the basis for a subchronic oral RfD for cylindrospermopsin, the panelists noted several concerns about this study. The study only examines male mice and seems to report differences in responses in different strains of the same mouse species. EPA needs to include an uncertainty factor to account for intraspecies variations. Another study, which dosed mice with a cultured material, found increases in male kidney weights, but this effect was not observed to the same degree in females. Because this study did not use a pure material, EPA should not use the study as the basis for a reference value. This study, however, highlights the concerns about differences in effects observed in males and females.

One panelist also noted that the kidney effects observed most likely represented a secondary effect of exposure. The liver is the primary target in mice. The panelists discussed the merits of using kidney versus liver effects as the point of departure. As a secondary effect, the effects on the kidney could occur at a higher dose than would affect the liver. In comparison to the kidney, the liver is quicker to regenerate after injury. As such, the kidney effects could be identified as

the more critical effect. The panelist discussed the mode of action of cylindrospermopsin and agreed that the liver was the primary target, however, existing research provides insufficient information to derive a reference value based on liver toxicity. The panelists recommended that EPA use the kidney effects as the point of departure for deriving the subchronic oral RfD, but EPA should clearly explain the confounding factors and concerns about this study in the toxicological review. The panelists also recommended that EPA add the recently published long-term study in mice exposed to bloom material (Sukenik et al., 2006[3]) to the toxicological review.

The panelists agreed that the benchmark dose (BMD) model used to derive the subchronic oral RfD for cylindrospermopsin was appropriate. The panelists noted that other BMD models would have produced similar endpoints and the BMD model was better than a NOAEL/LOAEL approach. To improve the description of the BMD model, EPA should include a detailed table of contents to link the text discussions to the appropriate appendices.

The panelists agreed that EPA should not use the 11-week gavage study in mice (Humpage and Falconer, 2003) to derive a chronic oral RfD for cylindrospermopsin. In addition, Sukenik et al. (2006) is also inappropriate for deriving a chronic oral RfD. This study examines effects resulting from exposure to bloom material and should only serve as a supporting study. One panelist noted that using the Sukenik et al. (2006) study would require an uncertainty factor of 10,000, which would be excessive for deriving a reference value even though EPA considers 10,000 to be a legitimate and usable uncertainty factor.

After reviewing the uncertainty factors applied to the subchronic oral RfD, the panelists agreed that a value of 1,000 was appropriate. One panelist noted that he did not fully agree with the rationale behind selecting the uncertainty factor, but that 1,000 was appropriate.

Discussion of Question 1: Cancer Assessment

In public comments submitted to EPA, Dr. Ian R. Falconer stated that available data are insufficient to draw conclusions about the carcinogenicity of cylindrospermopsin, but mechanistic evidence suggests a carcinogenic effect. The panelists agreed with this comment, noting that available information indicates that a cancer endpoint is a concern for cylindrospermopsin.

Discussion of General Charge Questions 1–3

The overall quality of the document is very good, however, the document is missing some relevant references, as noted in the discussion of charge questions 1–6.

In addition to the references provided, the panelists discussed research needs. They considered needs for acute, chronic, and cancer studies. One panelist felt that future studies should examine liver and kidney endpoints using biochemical and microscopy evaluations. If using

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[3] Cited in pre-meeting comments (Sukenik, A., Reisner, M., Carmeli, S. and Werman, M., Oral toxicity of the cyanobacterial toxin cylindrospermopsin in mice: long-term exposure to low doses, Environ Toxicol, 21(6), 575, 2006).

histopathology variables, a study should be limited to only 4 or 5 scientifically well-defined endpoints. Panelists also noted that local Departments of Health need answers to their concerns about reacting to blooms and protecting public health. Future research, such as a 96-hour mortality study, should be designed to support this need. The consensus statement for cylindrospermopsin, provided earlier in this section, summarizes and prioritizes the panelists' specific recommendations for additional research.

7. Microcystins

Consensus Statement

The expert panel prepared the following consensus statement regarding the microcystins toxicological review:

> **Acute Effects:** We request that EPA review the Fawell et al. (1999) for inclusion of the control groups. If necessary, EPA should request publication of this control group as an addendum in the same journal.
>
> **BMD Modeling:** The general consensus was that BMD modeling was correctly applied but that the description of how the points of departure were determined from those models should be more transparent.
>
> **Exposure:** The panel recognized that exposure to microcystins (e.g., through drinking water, algal supplements, food products, and recreational exposure, etc.) was considered not part of the hazard assessment and therefore beyond the scope of this document.
>
> **Carcinogenicity:** EPA needs to consider the conclusions of the International Agency for Research on Cancer (IARC) in that microcystins show a likely tumor promoter mechanism through their inhibition of PPIA 1 and 2A, and suggestive evidence from the epidemiological data from China, that lead to a conclusion that microcystins are "possibly carcinogenic to humans" (2B). In concurrence with IARC, the expert panel noted that there is suggestive evidence of carcinogenic potential for microcystin-LR in humans, and recommends that the cancer classification be changed to "suggestive evidence of carcinogenic potential."
>
> Due to a similarity of mechanism of the other microcystin congeners, it is likely that the other congeners will have carcinogenic potential.
>
> **Additional Studies:** The panel had the consensus opinion that the following are needed: properly designed acute oral studies for microcystins RR, YR, LA, LR, LF, and LW; pharmacokinetic data on the different congeners; properly designed short-term or subchronic study for LR; and, if resources are available, a chronic study for LR.

Discussion of Charge Questions 1 (RR, YR, and LA) and 1–6 (LR): Derivation of Reference Values

The panelists agreed that available toxicity information was inadequate for the derivation of oral reference values for microcystins RR, YR, and LA.

The panelists agreed that available toxicity information for microcystin-LR was inadequate for the derivation of an acute oral RfD. One panelist asked if the LOAEL from an acute study in mice (Ito et al., 1997) with an uncertainty factor of 10 could serve as the basis for an acute oral RfD. The other panelists indicated that the LOAEL was free-standing and the study did not meet minimum data reporting requirements. Another acute study in mice (Fawell et al., 1999) may be appropriate for deriving an acute oral RfD. Data from a control group, however, were lacking. The panelists recommended that EPA encourage the authors (Fawell et al., 1999) to republish the study findings and include results from controls, if possible.

A 28-day drinking water study in rats (Heinze, 1999) served as the basis for the short-term and subchronic oral RfDs for microcystin-LR. Concerns regarding this study, however, included the use of only two doses, potential inadequate pathology, use of rankings (e.g., moderate and severe) to describe effects, and the small study group size. The panelists discussed the applicability of a gavage study in mice (Fawell et al., 1999) and thought that this may be a better-designed study. Without appropriate controls, however, this study could not serve as the principal study for deriving a reference value. The panelists also noted that data from the 28-day drinking water study in rats (Heinze, 1999) identified a lower point of departure than the data from the gavage study in mice (Fawell et al., 1999). A lower and more protective reference value would result from using the 28-day drinking water study in rats (Heinze, 1999), and the panelists agreed that this study should serve as the principal study for the short-term and subchronic oral RfDs. One panelist suggested that EPA strengthen the rationale for selecting this study as the principal study.

In selecting the point of departure from the 28-day drinking water study in rats (Heinze, 1999), the panelists agreed that combining moderate and severe effects was appropriate. As described, the slight effects were not sufficiently different from the controls to ascribe them as an effect of microcystin-LR exposure. One panelist stated that more biochemistry effects than reported would have been expected for the severe effects group. One panelist noted that the original study data showed a low variability in responses at the high dose, and greater variability in responses at the low dose.

The panelists agreed that EPA selected and applied the BMD model appropriately. They recommended, however, that EPA strengthen the rationale for model selection (e.g., based on p-value and goodness of fit in conjunction with the Akaike Index). One panelist noted that the EPA RfDs differ from the World Health Organization (WHO) values, which may raise questions. Another panelist noted that the WHO assessment of microcystin-LR in drinking water and food has been under question recently.

An 18-month drinking water study in mice (Ueno et al., 1999) served as the basis for the chronic oral RfD. The panelists discussed several of the study shortcomings: use of only a single dose group, exposure to only female mice, and identification of only a free-standing NOAEL. One

panelist noted that a free-standing NOAEL may lead to the derivation of an RfD that is too conservative. Several panelists noted that other mice studies (Fawell et al., 1999) identified effects in males, but not females, which indicates that males may be more susceptible to microcystin-LR toxicity. After discussion, the panelists agreed that this study was suitable as the principal study for deriving a chronic oral RfD.

EPA included uncertainty factors of 1,000 to derive the short-term and subchronic oral RfDs for microcystin-LR. One panelist suggested reducing the uncertainty factor for these two RfDs to 300 by reducing the factor for database uncertainty from 10 to 3. (During discussions, Hawkins indicated that EPA limits uncertainty factors to values of 10, 3, and 0.) In examining other studies of microcystin-LR, reproductive toxicity appears to occur at higher exposure levels than those that cause liver toxicity. In considering the practical application of the RfDs, an uncertainty factor of 1,000 would result in RfDs lower than those for methylmercury, a known reproductive and developmental toxin. Two panelists wondered if the panel could recommend a higher uncertainty factor of 10,000 to address concern about tumor promotion. The panelists also discussed whether data from human exposures, which indicates that microcystin-LR has a similar mode of action in rodents and in humans, would support a lower uncertainty factor for animal-to-human extrapolation. The available human data include a number of confounding factors, such as exposure through the intravenous route to water containing microcystin from algal blooms. At the conclusion of the discussion regarding uncertainty factors, four panelists recommended that EPA apply an uncertainty factor of 300 for the short-term and subchronic oral RfDs. Two panelists recommended that EPA apply an uncertainty factor of 1,000.

Five of the six panelists agreed that EPA's use of an uncertainty factor of 1,000, which does not consider concerns about carcinogenicity, for the chronic oral RfD was appropriate. One panelist suggested adding an additional factor of 3 for a total uncertainty factor or 3000. This higher uncertainty factor would account for concerns about possible exposures through drinking water, food, and other sources, such as algal supplements.

The discussion of the uncertainty factor for the chronic oral RfD lead to a discussion about exposure through algal supplements. One panelist felt that algal supplements pose a hazard and EPA should mention these supplements as a possible exposure route. Another panelist noted that algal supplements are not pure compounds. Effects observed after exposure to an algal supplement would be equivalent to effects observed after exposure to algal extracts, which the panel deemed inadequate for deriving reference values. Another panelist thought that presenting the peak toxin values found in algal supplements would be misleading. These supplements would experience peak toxin occurrences similar to bloom events. Presenting even a median or mean detected toxin level would discount the potentially hundreds of samples with no toxins detected. During the discussion, Hawkins clarified that the toxicological review is not intended to detail possible exposure routes; rather the document should present the available toxicity data. As such, the panel recommended that EPA remove the reference to an unpublished study of food supplements fed to mice (Schaeffer et al., 1999).

Discussion of Question 1: Cancer Assessment

IARC recently completed an evaluation of microcystin-LR and classified this toxin as "Group 2B: the agent is possibly carcinogenic to humans." Hawkins noted that the IARC evaluation was

not available when EPA prepared the toxicological review for the microcystins. EPA would review this document when revising the toxicological review. The panelist recommended that EPA consider possible regulatory impacts or EPA requirements to correspond with IARC findings. Regardless of the IARC findings, the panelists believed that data were sufficient to indicate a carcinogenic potential based on mode of action and supporting data. The panelists recommended that EPA classify microcystin-LR as "suggestive evidence of carcinogenic potential."[4]

Discussion of General Charge Questions 1–3

The panelists agreed that the toxicological review for microcystins was well written and comprehensive.

The panelists discussed additional research needs. One panelist suggested that research should consider a possible toxicity equivalent approach if a similar mode of action could be identified among the microcystins. Regardless, understanding acute and short-term toxicity should be the priority. Other panelists agreed that future research should focus on acute toxicity. A panelist noted that studies of microcystin-LF have not been discussed and that the toxicological review excludes the non-polar microcystins. Another panelist noted that the toxicological review focuses on the four microcystins identified during the 2001 meeting. These are the four most common microcystins, and therefore understanding their toxicity would be most beneficial at this time. Another panelist noted that several studies of microcystin toxicity will be released soon and would provide additional data. The panelists also agreed that there was a need for a chronic study of microcystin-LR toxicity. The consensus statement for microcystins, provided earlier in this section, summarizes and prioritizes the panelists' specific recommendations for additional research.

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[4] During the review of the draft of this report, one panelist provided an additional reference, supplied by Dr. Ian Falconer, which clearly demonstrates the tumor promotion activity of microcystin-LR. Lian et al (2006) injected the carcinogen aflatoxin into wild mice and in transgenic mice carrying the X gene of hepatitis B. Dosing with microcystin-LR (10 micrograms per kilogram [ug/kg] weekly for 15 weeks) resulted in significant increases in liver tumors in both strains of aflatoxin-treated mice. At 24 weeks the liver tumor incidence in wild type mice dosed with aflatoxin and microcystin-LR was 19%, which is a threefold increase in tumorigenesis when compared to mice treated with aflatoxin alone (5%). This work provides further evidence of the carcinogenic potential of microcystin-LR.

Lian M, Liu Y, Yu S Z, Qian G S, Wan S G, Dixon KR (2006). Hepatitis B virus x gene and cyanobacterial toxins promote aflatoxin B1 induced hepatotumorigenesis in mice. World J. Gastroenterol. 12: 3065–3072.

Appendix A: Panelists

United States
Environmental Protection Agency
National Exposure Research Laboratory

External Peer Review Meeting of Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin, and Microcystins LR, RR, YR and LA

Andrew W. Breidenbach Environmental Research Center
U.S. Environmental Protection Agency
Cincinnati, Ohio
January 10, 2007

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Note: Dr. Stone is participating in this review as an independent expert, and not as a representative of the Oregon State Public Health Department.

Appendix B: Charge to Panelists

Charge to External Reviewers for the

Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin and Microcystins LR, RR, YR and LA

Introduction

The U.S. Environmental Protection Agency (EPA) is conducting a peer review of the scientific basis for the human health assessment of three cyanobacterial toxins: anatoxin-a, cylindrospermopsin and microcystins (LR, RR, YR and LA).

Feedback on the Toxicological Reviews of Cyanobacterial Toxins is currently being sought in three general areas: (1) general clarity and thoroughness of the documents, (2) issues concerning the derivation of reference values specific to these toxins, and (3) characterization of the carcinogenic potential of these toxins.

General Questions

1. Are the Toxicological Reviews logical, clear and concise? Has EPA accurately, transparently and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?

2. Are you aware of additional studies that should be considered in the assessment of the noncancer and cancer health effects of these toxins?

3. Please discuss research that you think would be likely to reduce uncertainties in the reference values for future assessments.

Questions Related to the Derivation of Reference Values for Anatoxin-a, Cylindrospermopsin and Microcystins (LR, RR, YR and LA)

Oral Reference Dose (RfD) Values

Anatoxin-a

1. The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute and chronic exposure durations. Do you agree with this conclusion? Is the rationale for not developing acute or chronic oral RfDs transparent and objective? If you do not agree with the conclusion that the data are inadequate to support derivation of oral RfD values for acute and chronic durations, and hence you conclude that derivation for acute and/or chronic derivations is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

2. The 28-day gavage study in mice (Fawell and James, 1994; Fawell et al., 1999) was selected as the basis for the short-term oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

3. In the 28-day gavage study selected as the basis for the short-term RfD, two potentially treatment-related deaths were reported. The original study authors concluded that the NOAEL for this study was 0.1 mg/kg-day based on these two deaths. This conclusion was based on their inability to determine the cause of death (i.e., to completely rule out a relationship with treatment), and they indicated that the true NOAEL may actually be 2.5 mg/kg-day.

Due to the low incidences of mortality (that showed no dose-response or gender consistency), the lack of characteristic clinical signs of acute neurotoxicity in the two animals that died, and the absence of toxicologically significant effects in the surviving mice, as well as the lack of effects at 2.5 mg/kg-day in mice reported in 5-day and developmental toxicity studies (Fawell and James, 1994; Fawell et al., 1999), EPA concluded that the deaths are likely to be incidental and that the actual NOAEL is 2.5 mg/kg-day. Is the designation of 2.5 mg/kg-day as the NOAEL for this study scientifically justified? Has the rationale for this designation been transparently and objectively described?

4. The 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980) was selected as the basis for the subchronic oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

5. Are the uncertainty factors applied to the points of departure (PODs) for the derivation of the short-term and subchronic RfD values scientifically justified and transparently and objectively described?

Cylindrospermopsin

1. The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute or short-term exposure durations. Do you agree with this conclusion? Is the rationale for not developing acute or short-term oral RfDs transparent and objective? If you do not agree with the conclusion that the data are inadequate to support derivation of oral RfD values for acute and chronic durations, and hence you conclude that derivation for acute and/or chronic derivations is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

2. The 11-week gavage study in mice (Humpage and Falconer, 2003) was selected as the basis for the subchronic oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

3. The critical effect identified in Humpage and Falconer (2003) was increased relative kidney weight. Is selection of this finding as a critical effect scientifically justified? Is the rationale for selecting this effect transparent and objective?

4. Benchmark dose (BMD) modeling was utilized to estimate a BMD and BMDL for increased mean relative kidney weight using 1 standard deviation above the control mean as the benchmark response level (BMR). After dropping the high dose group, the linear model adequately fit the data and produced an estimated BMD of 43.1 µg/kg-day and BMDL of 33.1 µg/kg-day. Was the modeling appropriately conducted and interpreted? The BMDL of 33.1 µg/kg-day was chosen as the POD for RfD derivation. Is the rationale for selecting this as the POD transparent and objective?

5. The conclusion was reached that the 11-week gavage study in mice (Humpage and Falconer, 2003) selected as the basis for the subchronic oral RfD study could not be utilized for the derivation of a chronic oral RfD due to the excessive uncertainty inherent in doing so. Do you agree with this conclusion? Is the rationale for not developing a chronic oral RfD transparent and objective? If you do not agree with the conclusion that the data are too uncertain to support derivation of oral RfD values for chronic durations, and hence you conclude that derivation for chronic derivations is appropriate, then please describe how you would recommend such derivation be completed and the rationale for such derivation.

6. Are the uncertainty factors applied to the point of departure for the derivation of the subchronic oral RfD scientifically justified and transparently and objectively described?

Microcystins (LR, RR, YR and LA)

1. The conclusion was reached that the available toxicity information was inadequate to support derivation of oral RfD values for microcystin-RR, -YR or LA. Do you agree with this conclusion? Is the rationale for not developing oral RfD values transparent and objective? If you do not agree with the conclusion that the data are inadequate to support derivation of oral RfD values for microcystin-RR, -YR or LA, and hence you conclude that derivation is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

Microcystin-LR

1. The conclusion was reached that the available oral toxicity information was inadequate to support derivation of an acute oral RfD value. Do you agree with this conclusion? Is the rationale for not developing an acute oral RfD transparent and objective?

2. The 28-day drinking water study in rats (Heinze, 1999) was selected as the basis for the short-term and subchronic oral RfDs. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

3. Liver toxicity observed by Heinze (1999) included liver lesions, serum enzyme changes and changes in relative liver weight. All three of these endpoints were considered for determining the point of departure for RfD derivation. BMD modeling was utilized to estimate a BMD and BMDL for each of these endpoints. Was the modeling appropriately conducted and transparently and objectively presented?

For the purposes of BMD modeling, the moderate and severe liver lesion categories reported by Heinze (1999) were combined and the BMDs and BMDLs for these lesions estimated by the log probit model were 11.0 and 6.4 µg/kg-day, respectively. The BMDL of 6.4 µg/kg-day was chosen as the POD for RfD derivation. Is the rationale for selecting this as the POD transparent and objective? Is selection of liver lesions as a critical effect scientifically justified? Is combining the moderate and severe lesions scientifically justified? Is the rationale for selecting this effect transparent and objective?

4. Data from the 90-day gavage study in mice conducted by Fawell et al. (1999) was considered for the derivation of the subchronic RfD. The BMDL from the Heinze (1999) study (6 µg/kg-day) is approximately an order of magnitude lower than any of the BMDL values derived from endpoints from the 90-day gavage study (57-66 µg/kg-day) therefore, the BMDL of 6 µg/kg-day from Heinze (1999) was chosen as the POD for subchronic RfD derivation. Do you agree with this decision? Is the rationale for selecting 6 µg/kg-day as the POD transparent and objective?

5. The 18-month drinking water study in mice (Ueno et al., 1999) was selected as the basis for the chronic oral RfD. This study used only a single dose level and identified a freestanding NOAEL but was chosen for RfD derivation because it was a well-conducted study of chronic duration and employed a relevant exposure route (drinking water). Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

6. Are the uncertainty factors applied to the points of departure for the derivation of the short-term, subchronic and chronic oral RfDs scientifically justified and transparently and objectively described?

Questions Related to the Cancer Assessments for Anatoxin-a, Cylindrospermopsin and Microcystins (LR, RR, YR and LA)

1. Do the available data support the conclusion that the database for each of these toxins provides inadequate information to assess carcinogenic potential based on the weight-of-evidence categories in the EPA 2005 Guidelines for Carcinogen Risk Assessment? Please describe the basis for your view.

Appendix C: Observers

External Peer Review Meeting of Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin, and Microcystins LR, RR, YR and LA

Andrew W. Breidenbach Environmental Research Center
U.S. Environmental Protection Agency
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January 10, 2007

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Appendix D: Agenda

United States
Environmental Protection Agency
Office of Research and Development

External Peer Review Meeting of Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a, Cylindrospermopsin, and Microcystins LR, RR, YR and LA

Andrew W. Breidenbach Environmental Research Center
U.S. Environmental Protection Agency
Cincinnati, Ohio
January 10, 2007

Agenda

9:00 a.m. **Opening Remarks and Introductions**..... *Jan Connery, ERG, Facilitator*

9:15 a.m. **Observer Comment Session**.....*Jan Connery*

9:30 a.m. **Reviewer Discussion of Anatoxin-a Document** ..... *Daniel Dietrich, Panel Chair*
- Overview of Charge Questions and Discussion Areas
- Discussion of Questions 1-5 Related to the Derivation of Reference Values for Anatoxin-a
- Discussion of Question 1 Related to the Cancer Assessment for Anatoxin-a
- Discussion of General Questions 1-3 for Anatoxin-a
- Development of Conclusions and Recommendations

10:30 a.m. BREAK

10:45 a.m. **Reviewer Discussion of Cylindrospermopsin Document** .....*Daniel Dietrich*
- Overview of Charge Questions and Discussion Areas
- Discussion of Questions 1-6 Related to the Derivation of Reference Values for Cylindrospermopsin
- Discussion of Question 1 Related to the Cancer Assessment for Cylindrospermopsin
- Discussion of General Questions 1-3 for Cylindrospermopsin
- Development of Conclusions and Recommendations

12:15 p.m. LUNCH

1:15 p.m. **Reviewer Discussion of Microcystins (LR, RR, YR and LA) Document**.....*Daniel Dietrich*
- Overview of Charge Questions and Discussion Areas
- Discussion of Questions 1 (for LR, RR, YR and LA) and 1-6 (for LR) Related to the Derivation of Reference Values for Microcystins

2:45 p.m. BREAK

3:00 p.m. **Reviewer Discussion of Microcystins Document (continued)** *.....Daniel Dietrich*

- Discussion of Questions 1-6 (for LR) Related to the Derivation of Reference Values (cont.)
- Discussion of Question 1 Related to the Cancer Assessment for Microcystins
- Discussion of General Questions 1-3 for Microcystins
- Development of Conclusions and Recommendations

4:45 p.m. **Concluding Remarks**

5:00 p.m. ADJOURN

Appendix E: Presentation by Belinda Hawkins, EPA

[PowerPoint slides provided as a PDF file]

Appendix F: Presentation by Daniel Dietrich, Peer Review Panel Chair

[PowerPoint slides provided as a PDF file]

Appendix G: Panelists' Pre-Meeting Comments

Panelist Biographies

Gregory Boyer

Dr. Boyer received a BS in 1975 from the University of California-Berkeley, and a Ph.D. in Biochemistry in 1980 from the University of Wisconsin-Madison. He is currently a Professor of BioChemistry at the State University of New York, College of Environmental Science and Forestry (SUNY-ESF) in Syracuse NY. Dr. Boyer has 30 years experience in algal toxin analysis, and since 2002 has been a Principal Investigator (PI) for a NOAA MERHAB regional project to develop a tier-based monitoring system for cyanobacterial toxins in the lower great lakes. He is also the lead scientist on the SUNY-ESF source sentinel consortium to develop remote monitoring systems for protection of municipal water supplies. Additionally, he is Director of the SUNY-ESF algal toxins project and lead PI on the toxin analysis laboratory, a full service facility for the chemical analysis of marine and freshwater algal toxins. Dr. Boyer has written more than 75 peer-reviewed publications on algal, fungal and plant natural products. He is a recipient of the State University of New York Research Foundations 2003 Award for Excellence in the Pursuit on Knowledge and the 2005 ESF award for integrating outreach activities with Teaching and Research.

Wayne Carmichael

Dr. Carmichael received his Ph.D. from the University of Alberta, Edmonton, Alberta, Canada in 1974. He is currently Professor of Aquatic Biology/Toxicology and Director of the Environmental Sciences Ph.D. Program-Department of Biological Science at Wright State University in Dayton, Ohio. His current research interests with regard to blue-green algae (cyanobacteria) toxins (biotoxins and cytotoxins) includes:

1. Collection, isolation and culture of freshwater and marine toxic strains.
2. Large scale culture techniques for toxic strains.
3. Isolation and chemical characterization of pharmacologically active compounds.
4. Development of detection methodologies for cyanotoxins
5. Toxinology and pharmacology.
6. Induction and regulation of pharmacologically active compounds.
7. Risk assessment and management of cyanobacteria and their water-based diseases

Daniel Dietrich

Daniel Dietrich, born 1959 and citizen of Switzerland is a professor of toxicology, head of Human and Environmental Toxicology at the University of Konstanz, Germany, and an adjunct professor at the University of Pittsburgh, PA. He received his PhD in toxicology from the Swiss Federal Institute of Technology (ETH) in Zurich Switzerland in 1988. He is a member of the Society of Toxicology (SOT), Society of Environmental Toxicology and Chemistry (SETAC), International Society of Toxinology (IST), European Society of Toxicology (Eurotox), Fellow of the Academy of Toxicological Sciences, and until November 2006 served as adjunct expert judge at the Swiss Supreme Court in Bern, Switzerland. He has been conducting research concerned with human and environmental risk assessment in conjunction with cyanobacterial toxins, mycotoxins, and industrial chemicals. He has published over 100 original peer-reviewed articles and bookchapters. He has served and is still active on numerous international review and validation management panels, the most recent ones in June 2006, in Lyon France at the IARC/WHO review of cancer risks from nitrite, nitrate, microcystin-LR and nodularin and the OECD VMG-Non Animal for Endocrine Disruption in Tokyo, Japan December 11-14, 2006. Current research interests span from toxicokinetics of microcystin congeners and microcystin toxicology in general to renal carcinogenesis and the progression of pre-neoplastic lesions to veritable tumors.

Maria Runnegar

Dr. Runnegar received a Ph.D. in BioChemistry from the University of Queensland in Brisbane, Australia in 1968. She is currently an Associate Professor of Research Medicine at he Keck School of Medicine at the University of Southern California. Before 1987, Dr. Runnegar worked in Australia at the University of New England investigating the occurrence of cyanobacterial blooms (Microcystis, Cylindrospermopsis, Nodularia and Anabaena) and their toxicity in a number of experimental animals including mice, rats, sheep and chickens. Acute, subacute and chronic dosings were investigated. In vitro studies included the isolation and characterization of microcystin and nodularin, and the investigation of the mode of action of these hepatotoxins. Since 1987 at the University of Southern California she has been studying the mode of action of microcystins, cylindrospermopsin and synthetic analogs and okadaic acid with emphasis on the sublethal effects of the toxins in vivo and in vitro in a number of cell types and subcellular preparations.

Dave Stone

Dr. Dave Stone is a public health toxicologist employed by the Oregon Public Health Division. Dr. Stone received his Ph.D. in Environmental & Molecular Toxicology from Oregon State University in 2002. His areas of expertise include risk assessment, risk communication and the toxicology of biological toxins, heavy metals and pesticides. Dr. Stone is the lead consultant for fish, cyanobacteria and drinking water health advisory programs in Oregon. Dr. Stone has served on the USEPA Harmful Algal Bloom-Cyanobacteria expert panel, the Oregon Air Toxic Science Advisory Committee and the Oregon-Native American Fish Consumption Advisory Group. He is a member of the Society of Environmental Toxicology and Chemistry, the Society for Risk Analysis and the Oregon Collaborative on Health and the Environment.

Christopher Williams

Dr. Williams is currently the President of GreenWater Laboratories/CyanoLab, a private analytical laboratory (2001-present) that specializes in the identification and quantification of freshwater algae and cyanotoxins. He received his Ph.D. in Aquatic Toxicology from the Virginia Institute of Marine Science at the College of William and Mary (1995) and performed post-doctoral fellowships at both Yale University (1995-96) and at the Whitney Molecular Marine Laboratory, University of Florida (1996-98). Dr. Williams is a member of the Florida Harmful Algal Bloom Task Force and its associated Public Health Technical sub-committee. He was the project coordinator for the Cyanobacteria Survey Project (1999-2001), a Florida statewide assessment on the prevalence and distribution of potentially toxigenic cyanobacteria and cyanotoxins. Dr. Williams and GreenWater Laboratories/CyanoLab has assisted and performed analyses for the Center for Disease of Control, state Environmental Protection Agencies, the Florida Water Management Districts, and the Florida Department of Health/Aquatic Toxins Program in identifying and measuring cyanotoxins in both environmental and drinking water resources. Dr. Williams has been an invited speaker to discuss and explain current cyanotoxin data at both national and international conferences conducted by the North American Lake Management Society and the American Water Works Association/Water Quality Technical Conference.

Gregory Boyer

Anatoxin-a

General Questions

1. Are The Toxicological Reviews Logical Clear And Concise?

Yes. However under human studies 4.1 (p5): I think the general consensus in the scientific community is that the initial report by the coroner was in error, and that anatoxin-a was not the responsible agent for death of the Wisconsin teenager. This report should not perpetuate that myth.

2. Are Additional Studies Needed?

The NOAEL and FEL in mice are pretty convincing, but the lack of other animal models is worrisome. Toxicity testing in a second species is needed. The report effects from laboratory studies are often at odds with the measured concentrations in natural samples attributed to animal fatalities. Either there is a large interspecies variability, rapid degradation of anatoxin-a under natural conditions, or some other factor at play.

3. What Research Is Needed To Reduce Uncertainties In The Reference Values?

Additional animal studies and toxicological effects of its two "metabolites": anatoxin-a epoxide and the dihydro anatoxin-a derivative.

Missing Information in Report:

Metabolism studies on anatoxin-a in mussels have been done by Schwalbe and Pflugmacher[5] suggesting that it is metabolized to the epoxide derivative in *Dreissena.*

Specific questions:

1. Unable To Derive An Acute Oral RfD: While I agree that the available studies are limited due to single animal and small sample sizes, anatoxin-a appears to show a very tight threshold between its NOAEL, LOAEL and LD50. In that sense, the LOAEL may be very close to the current NOAEL derived from short term studies and, given that most of the mice died within the first couple of hours, the data from the short term studies may be appropriate and provide a sufficient margin of safety for an acute RfD. The acute RfD should be very close to that derived for the short term RfD and, if anything, using the short term RfD would provide an additional margin of safety. The lack of animal studies other than in mice is more worrisome but that could be accounted for though the uncertainty factors. The sub chronic data appears to show that no are no long term impacts at the doses tested and could be used to estimate the chronic RfD, especially if one increased the margin of safety in the uncertainty factors. It is highly unlikely that a population will suffer from a chronic exposure to anatoxin-a, given the episodic nature of the toxigenic events.

2, 3 Is the 28-Day Gavage Study Appropriate For The Short Term RFD? Yes, based on the lack of any chronic effects. I agree with EPA in that the two observed fatalities were likely to be incidental to the treatment and could be eliminated from the data.

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5 Schwalbe, H., and S. Phlugmacher (2002) Comparison of the detoxication from a cyanobacteria hepatotoxin microcystin-RR) and a neurotoxin (anatoxin-a) in the freshwater mussel Dreissena polymorph. Abstracts, XHAB. St Petersburg, FL October 2002.

4. Selection Of The 7 Day Sub Chronic Study. This is an area where more work is needed. You can justify selection of the 7-day sub chronic study based on the observation that it is the best study available, but the study has several defects, namely limited dose levels and lack of a suitable marker for exposure.

5. Uncertainty factors: These are clearly defined but should be increased for the sub chronic studies due to lack of data.

Cylindrospermopsin

General Questions

1. Are the toxicological reviews logical clear and concise?

Ohtani et al (1992) initially reported the "S" conformation for the natural form of cylindrospermopsin based upon their nmr studies. Subsequent isolations of the toxin from *Umezakia natans* (Harada et al, 1994) and the isolation of both the toxin and its 7-hydroxy epimers from *Aphanizomenon ovalisporum* (Banker et al, 2000), did nothing to either confirm or change this assignment. A recent total synthesis of cylindrospermopsin, controlling all the steriochemical centers, found the product prepared with the "S" configuration at C-7 was identical with 7-epi-cylindrospermopsin (Heintzelman et al, 2001). This "S" conformer has been assigned to 7-epi-cylindrospermopsin (CA registry number 265652-18-4) and by inference; the "R" isomer at C-7 has been assigned to cylindrospermopsin (CA registry number 10143545-90-8). The original structure as reported by Ohtani et al. (1992) and cited in this document is actually incorrect. The structure as shown in the document is correct.

Page 26 line 46 contains a suspected typo. I think they mean Table 4-2.

They should standardize their units as either ug/kg-day or mg/kg-day but not mix them.

2. Are additional studies needed?

3. What research is needed to reduce uncertainties in the reference values?

One of the main limitations of this work seems to be the total dependence on the mouse as a model system. Additional studies in other species are needed. A clear identification of biological target would also help in understanding the toxicological experiments.

Specific questions:

1. They choose not to use the available data from the cell extract studies. I think this should be looked at again in reference to developing an acute RfD. I would be less comfortable in using this for subacute and chronic studies, but the 4 independent acute trials using cell extracts seem to agree within a factor of 2.

2. The Humpage and Falconer study is well documented and appropriate.

3. The conclusion that kidney weight increased is weak. At best, it is consistent with the idea that cylindrospermopsin inhibits protein systems, and the kidney is increasing its mass to make up for that lost capacity. Definitive experiments looking at changes in kidney protein biosynthesis would help in this regard.

4. The rational was well described. I am not qualified to comment on the pros and cons of BMD modeling.

5. The main objection to using the 11 week study was that the uncertainty factors would be greater than 10,000. While I understand the authors hesitation, I think the 11-week study could be used to estimate a RfD as was done by the authors of that study.

6. Uncertainty factors are the standard ones and generally acceptable.

Microcystins LR, RR, YR and LA

General Questions

1. Are the toxicological reviews logical clear and concise?

Much of the early work refers to a toxin extracted from M. aeruginosa at MW 1019. While this could refer to Microcystin-YM (m/z 1019), it may also refer to a variant of the protease inhibitor Cyanopeptolin A, or be a misidentified sodium adduct (+22) of microcystin LR (m/z 1017). I think more emphasis should be given to the compounding and conflicting effects of the other bioactive peptides (microviridins, cyanopeptolins, etc) that may be present in these toxin blooms.

Planktothrix needs to be added to the list of producing species on pages 2 and 3.

2. Are additional studies needed?

The colorectal cancer exposure studies in Zhejiang Province are probably suspect due to the complicating factors from the high levels of aflatoxin that exist in foodstuffs in that province[6]. Additional long term epidemiological studies are needed to evaluate the carcinogen risk assessment of the microcystins (and cylindrospermopsin).

3. What research is needed to reduce uncertainties in the reference values?

Additional studies in species other than mouse or rat would be beneficial as the transport of this toxin greatly affects its pharmacokinetics.

Specific questions:

Microcystin-LR

1-6 I agree with the choices made in this review. I don't think they have enough data to develop an acute oral RfD based on the information provided, though they could certainly estimate one using the lower dose levels of the four studies. Neither do I think they can use the short term study of Heinze to develop an acute RfD. The choice of the 28-day drinking water study by Heinze is logical for the short term exposure, as was the choice of the three indicator endpoints. I cannot evaluate if the model was run appropriately, though it seems logical to combine the moderate and severe liver lesions categories. Using the more conservative value of 6 ug/ kg-day also provides for an additional margin of safety in the sub chronic study; however the NOAEL value of 40 ug / kg-day from the 90 day study has considerable historical acceptance in the literature and by the World Health Organization. All the choices were clear and transparent, the uncertainty factors applied are certainly reasonable, though there is little in the way of scientific justification for any particular value other than general use.

Cancer Assessment

There is adequate (and conflicting) information to properly assess the carcinogenic potential of both cylindrospermopsin and microcystins, though an argument could be made that anatoxin-a has no carcinogenic behavior based on its mode of action and lack of any evidence to the contrary.

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6 Comparison of three methods for screening aflatoxin-producing strains. Wang Z G, Hangzhou Zhonghua yu fang yi xue za zhi [Chinese journal of preventive medicine] (1990), 24(5), 287-9.

Wayne Carmichael

Review comments for: Toxicological Reviews of Cyanobacteria Toxins: Anatoxin-a, Cylindrospermopsin and Microcystins LR, RR, YR and LA

December 30, 2006

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Charge Issues/Questions: Conduct a peer review of the draft documents for Antx-a, CYN and Mcyst-LR,RR,YR and LA, submit written comments, read and incorporate any public comments, actively participate in a 1-day peer review workshop, review final draft of the peer review.

Public comments: no public comments have been received to date. Comments outside this public comments request were received from Prof Ian Falconer. These are:
"Thank you for the opportunity to comment on the EPA toxicological reviews of anatoxin-a, cylindrospermopsin and microcystin.

**I have no comments on anatoxin-a, it is a good clear account. **Cylindrospermopsin -as follows This is an impressive review of the relevant data, silence on my part reflects agreement with the material.

--page 7. Palm Island poisoning. Section 4.1 lines 13,28. The individuals poisoned initially showed constipation, and only 2-3 days later had profuse bloody diarrhea. This rules out copper poisoning which is immediately followed by diarrhea. this event is discussed in my book pages 88-90.

--page 8 line 4. My examination of the histopathology did not conclusively demonstrate whether the main liver pathology was caused by cylindrospermopsin or microcystins. It has proved difficult to access the slides, so it is unanswered. Overall my opinion is that the pathology shows a combination of both toxins, and not microcystin alone. This is discussed in pages 87-88 in my book.

Reviewer note: CYN was not found in serum or liver but it was confounded by the methods of analysis available at the time of the outbreak. CYN was identified in the water filtration system (see Carmichael et al 2001). Dr Falconer is the only one who has expressed an opinion that the limited pathology available indicates both toxins contributed to the poisonings. In 2006 this reviewer spent 1 month in Recife-Pernambuco-Brazil on a follow-up review of Caruaru patient health status. I also attempted to engage the liver specialist (Dr Vittorino S. Barretto) to compile the histopathology data and publish on this event. He agreed it should be done but would not agree to a schedule for publishing. The available pathology and histopathology does support Microcystin exposure but other pathology esp fatty liver supports exposure from another toxin and therefore CYN could be involved.

--page 13 section 4.3 A study on developmental effects of cyn has recently been completed by Dr. Neil Chernoff, in the EPA at Research Triangle Park. It It may be possible for you to access it.

--page 24 lines 10-12 and 22-23 This is misleading. In the preliminary carcinogenicity study no controls showed neoplasia, whereas 5 of the cyn treated mice had neoplasms. The relative risk of 6.2 was not significant due to limited numbers of animals. See my book page 105. I agree that no conclusion can currently be drawn on the carcinogenicity of cyn, but there is very suggestive mechanistic evidence, and this preliminary study, pointing towards carcinogenicity. I have the cancer data for the population poisoned in Palm Island, and for a comparable indigenous community, which shows raised rates of gastrointestinal cancers in Palm Island people. This is unpublished and needs more evaluation, I am mentioning it as it also points towards cyn as a carcinogen.

reference 'Cyanobacterial toxins of drinking water supplies; cylindrospermopsins and microcystins' Falconer , Ian R., CRC Press, 2005.pp.279.

**Due to the length of the microcystin data, it will be the New Year
before I have time to comment." –Ian Falconer

General Questions:
A) Are the toxicological reviews logical, clear and concise?
**Anatoxin-a
This review is especially clear and concise. Due to the lack of studies on toxicokinetics and hazard evaluation the conclusions that oral RfD's cannot be derived is acceptable.

A few additions and edits will make this review even more clear and accurate:
a) page2- In 2004 a data base of all cyanotoxin and CyanoHAB ref was done for the EPA by WWC. The contractor produced a compact disk containing all references pertaining to toxic cyanobacteria in freshwater or drinking water (approximately 4000 to Jan 2004). These references were put in the program Procite. The data base is found on the EPA website and is titled CyanoHAB Search: A list of Toxic Cyanobacteria References. It should be included in this document preparation.

b) page 3-It is this reviewers suggestion that only genera/species of cyanobacteria confirmed to produce a cyanotoxin from established laboratory cultures be included in a reference to genera/species that produce a cyanotoxin. In some cases where a waterbloom that is essentially a mono species or genus and is shown to produce a cyanotoxin could be cited. In this text the ref to Microcystis producing Anatoxin-a would not qualify since there are no cultures of Microcystis that produce this toxin. The ref to this genus producing Anatoxin-a is from mixed blooms (most likely) and was not confirmed.

c) page 6- Suggest adding the recent ref (Toxicon 2005??) from France on dog poisonings from Antx-a where Phe was also present and discussed as a confounder in diagnosing Antx-a poisoning.

d) page 22- There is some work by USAMRID on inhalation toxicity--I think Antx-a was included. Check CyanoSearch data base

e) page 27- If this is the ref to a teenage Wisconsin boy--please verify genera present quote. This reviewer was told no cyanobacteria were identified in the suspect source water (a golf course water hazard).

f) page 27-uncertainty

g) page 28-Carmichael and Gorham---Infernal. should be Internal.

**Cylindrospermopsin
This review is well written and understandable. Conclusions regarding RfD's are acceptable. Some edits for the document are given below and would improve its accuracy and usefulness.

a) page 2-In 2003 the EPA contracted with this reviewer to data base all ref for toxic cyanobacteria. This was done and placed on a CD using Procite software. The data base has over 4000 ref and is located on the EPA website-it is titled CyanHab Search a list of toxic cyanobcteria references. It should be included in this relevant literature search.

b) page 3- In 2004 the EPA contracted with this reviewer for production of benchmark standards of CYN. This was done and delivered to the Cincinnati lab in 2004. Perhaps this should be mentioned in this review?

**Microcystins-LR,RR,YR and LA
a) page 2- Include EPA data base citation from Carmichael 2004

b) page 3- Insert reference for number of Microcystin congeners or better yet a table of all congeners

c) page 35- Please check EPA data base-I believe this South African work with vervet monkeys was published

d) page 39- Although the toxin was not specified an early reproductive study on rats by Sirenko et al (Ukraine) was published in the book by Carmichael (1981)

e) page 41- CDC Surveillance Summaries Dec 22, 2006 (55(SS12) reports: During 2004, two toxin-associated outbreaks occurred in untreated water venues in Nebraska. These outbreaks were attributed to elevated levels of microcystin toxin (17) from blue-green algae (i.e., cyanobacteria) in lakes, causing 22 cases of illness. The predominant illnesses in both outbreaks involved dermatitis and gastroenteritis. Patients who sought medical care had a combination of rashes, diarrhea, cramps, nausea, vomiting, and fevers.

f) page 42- This study did not provide follow-up info but mainly repeat info of the Jochimsen work. It provided more info on one case study where hemoperfusion was attempted (without success) to remove circulating microcystins.

g) page 43- In a recent study (Yuan et al 2006) follow-up analysis of serum and liver was done 10 years after the initial exposure. This information would be important to summarize here.

h) page 67- Include Lankoff et al ref 2004--Toxicology for other immune effect results.

i) page 68- Include recent papers by Lankoff et al (Mutagenesis) 2006 for more info on this topic.

j) page 79- Include conclusions from the international workshop (Europe-2006) on cancer risk classification where Microcystins are given a cancer risk classification.

k) page 105- Hilborn et al ref-replace with published ref by these authors (2005 and 2006)

l) page 112- Runnegar et al 1981-ref-spelling-change Deformastion to Deformation

B) Additional studies that should be considered in the assessment of the noncancer and cancer hazards.

**Anatoxin-a—The available toxicology and mechanism of action for Antx-a argues that additional studies for cancer hazard are not needed. Because Antx-a is a potent Ach mimic there is a risk for subchronic neurodegenerative toxicology and research to support this work should be considered.

**Cylindrospermopsin-the toxicology of Cyn is overall poorly understood. Studies to derive the chronic and acute mechanisms, including carcinogenicity and genotoxicity, should be done.

**Microcystins-LR,RR,YR and LA-The basic acute mechanism of Microcystins is understood well. The research need is for studies on the subchronic, carcinogenicity and genotoxicity. Use of Mcyst-LR is the most appropriate congener for all of these studies as regards US water risk-but not necessarily for other countries. This reviewer feels that if the 4 Mcyst congeners are used for toxicology studies to determine RfD's for acute and chronic toxicity the results will be fully comparable with all the Mcyst congeners.

C) Research to reduce uncertainties in the reference values.

Available data for calculation of RfD's for the three toxin groups, are in general insufficient. The writers were able to derive short term and subchronic oral RfD's for Antx-a but not acute and chronic oral RfDs. Inhalation RfC's and carcinogenicity were not derived. For Cyn derivation of acute, short term and chronic oral RfD's could not be done due to insufficient data. A subchronic oral RfD was derived and the data do seem appropriate for this conclusion. For the Mcysts the available data allowed a derivation or oral short-term, subchronic and chronic Rfd's for one of the Microcystin congeners—Microcystin-LR (which is also the most commonly found congener in US waters—but not necessarily in other geographies i.e. Australia). Inhalation RfC's and carcinogenicity of Microcystins was not determined.

The inability of available data to allow more RfD's to be determined for all three groups of cyanotoxins argues for more research to allow reduction of uncertainties in the available data and hence derivation of the needed RfD's. A major step needed to accomplish this research is the availability of reference standards in the quantities needed for the toxicology work. This can be best accomplished by supporting the medium scale culture (hundreds of liters) of cyanobacteria that produce Antx-a, Cyn, Mcyst-LR, RR, YR and LA followed by the purification and certification of the toxins plus a distribution arrangement for their safe use in the toxicology. In order to develop a safe distribution system some consideration needs to be given to working with the guidelines and provisions for the biohazards since Microcystins are on the CDC select agent list and are regulated by the new Dept of transportation regulations.

Questions related to derivation of reference values for the three cyanotoxin groups.
This reviewer is in agreement with the writers oral reference dose (RfD) values determined for the three cyanotoxin groups-i.e., Anatoxin-a, Cylindrospermopsin and Microcystin-LR, RR, YR and LA.

Supplemental material for: <u>Review comments for: Toxicological Reviews of Cyanobacteria Toxins: Anatoxin-a, Cylindrospermopsin and Microcystins LR, RR, YR and LA</u>
Wayne Carmichael
January 7, 2007

A) Are the toxicological reviews logical, clear and concise

**** Anatoxin-a:**
a) page 2-
The below is a web location for the EPA data base on toxic cyanobacteria referred to on page two of each review document.
Subject: RE: CyanoHAB ref data base

The Cyanobacteria literature list is at the following web address:

http://www.epa.gov/safewater/standard/ucmr/main.html#meet

It can be found by going to www.epa.gov/safewater and looking on the left side of the page for "regulations and guidance". In the regulations and guidance page, there is a section on guidance and policy documents. Under "guidance and policy documents" look for "Unregulated Contaminant Monitoring Regulation" and under this look for "additional EPA supported meetings and materials". The five parts of the literature list are attached separately.

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b) page 3.-In the two ref listed in the review-i.e. Fawell et al. 1999 and Viaggiu et al. 2004—Microcystis is not listed as producer of anatoxin-a.

c) The French ref is: **First report in a river in France of the benthic cyanobacterium Phormidium favosum producing anatoxin-a associated with dog neurotoxicosis**
Muriel Guggera,*, Se´verine Lenoira,b, Ce´line Bergera, Aure´lie Ledreuxa,
Jean-Claude Druartc, Jean-Franc¸ois Humbertc, Catherine Guettea, Ce´cile Bernarda. A USM0505 Ecosyste`mes et interactions toxiques, M.N.H.N., 12 rue Buffon, 75005 Paris, France bUnite´ Toxines, Polluants Organiques et Pesticides, A.F.S.S.A., 23 avenue du Ge´ne´ral de Gaulle, 94706 Maisons-Alfort Cedex, France cI.N.R.A.-UMR CARRTEL, B.P. 511, 74203 Thonon-les-Bains Cedex, France

d) page 22- USABRDL report 9120-Sept 1993. Treatment for removal of biotoxins from drinking water. This report does not include ref to inhalation toxicology for Antx-a. This reviewer is incorrect in thinking some work had been done by USAMRID.

e) page 27—The confusion surrounding this possible acute-lethal poisoning by anatoxin-a needs to be discussed in this document as an unconfirmed and unlikely example of CyanoHAB poisoning.

****Microcystins-LR, RR, YR and LA**

c) no reference was found for the S.A study

ref for reproductive work: Yu,A, Kirpenko, L.A., Sirenko, N.I. Kirpenko. 1981. Some aspects concerning remot after effects of Blue-green Algae toxin impact on animals. In Carmichael, WW (ed) 1981. The Water Environment: Algal Toxins and Health. Plenum Pres. Pp.257-270.

g) Ref for follow-up human analysis-Brazil: Yuan,M. **W. W. Carmichael**, E. D. Hilborn. 2006. Microcystin Analysis in Human Sera and Liver from Human Fatalities in Caruaru, Brazil 1996. Toxicon. 48:627-640.

h) immune effects: Lankoff, A., **Carmichael, W.W**., Grasman, K.A., and Yuan, M. 2004. The uptake kinetics and immunotoxic effects of microcystin-LR in human and chicken peripheral blood lymphocytes *in vitro*. Toxicology. 204:23-40.

genotoxicity: Lankoff A., J. Bialczyk, D. Dziga , **W. W. Carmichael,** I. Gradzka, H. Lisowska, T. Kuszewski, S. Gozdz, I. Piorun, A. Wojcik. 2006. The repair of gamma-radiation induced DNA damage is inhibited by Microcystin-LR, the PP1 and PP2A phosphatase inhibitor. Mutagenesis 21(1): 83-90.

Lankoff, A., J. Bialczyk, D. Dziga, **W. W. Carmichael**, H. Lisowska, A. Wojcik. Inhibition of nucleotide excision repair (NER) by the PP1 and PP2A inhibitor-microcystin-LR in CHO-K1 cells. 2006.Toxicon. 48:957-965.

j) ref for the carcinogenicity of microcystins published by the International Agency for Research on Cancer-which met in Lyon, France-June 2006 is: **Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Web reference- http://oncology.thelancet.com Vol 7 August 2006. (see attached pdf)**

References:

Carmichael, W.W., M. Stukenberg. 2005. North American CyanoHABs. In. Codd, G.A., S.M.F.O. Azevedo, S.N. Bagchi, M.D. Burch, W.W. Carmichael, W.R. Harding, K. Kaya, H.C. Utkilen. 2006. CYANONET, a Global Network for Cyanobacterial Bloom and Toxin Risk Management: Initial Situation Assessment and Recommendations. UNESCO/IHP-Paris: Working Series #SC-2005/WS/55.

Carmichael, W.W., Stukenberg, M.C. 2006 Blue-green algae (Cyanobacteria). In: Encyclopedia of Dietary Supplements 2nd Edition. 2006. Coates, P.M., Blackman, M.R., Cragg, G.M., Levine, M., Moss, J., White, J. (eds.) New York: Marcel Dekker, Inc. (a div. of) Taylor and Francis Books.ISBN# 0-8247-5504-9 (paper) ISBN# 0-8247-5503-0 (electronic).

Codd, G.A., S.M.F.O. Azevedo, S.N. Bagchi, M.D. Burch, **W.W. Carmichael,** W.R. Harding, K. Kaya, H.C. Utkilen. 2005. CYANONET: A Global Network for Cyanobacterial Bloom and Toxin Risk Management: Initial Situation Assessment and Recommendations. UNESCO/IHP-Paris: Working Series #SC-2005/WS/55.

Hilborn, E.D., **Carmichael, W.W**., Yuan, M., Azevedo, S. M.F. O. 2005. A simple colorimetric method to detect biological evidence of human exposure to microcystins. Toxicon. 46: 218-221.

Lankoff, A., **Carmichael, W.W**., Grasman, K.A., and Yuan, M. 2004. The uptake kinetics and immunotoxic effects of microcystin-LR in human and chicken peripheral blood lymphocytes *in vitro*. Toxicology. 204:23-40.

Lankoff A., J. Bialczyk, D. Dziga , **W. W. Carmichael,** I. Gradzka, H. Lisowska, T. Kuszewski, S. Gozdz, I. Piorun, A. Wojcik. 2006. The repair of gamma-radiation induced DNA damage is inhibited by Microcystin-LR, the PP1 and PP2A phosphatase inhibitor. Mutagenesis 21(1): 83-90.

Lankoff, A., J. Bialczyk, D. Dziga, **W. W. Carmichael**, H. Lisowska, A. Wojcik. Inhibition of nucleotide excision repair (NER) by the PP1 and PP2A inhibitor-microcystin-LR in CHO-K1 cells. 2006.Toxicon. 48:957-965.

Ott, J.L. and **W. W. Carmichael**. 2006. LC/ESI-MS Method Development for the Analysis of Hepatotoxic Cyclic Peptide Microcystins in Animal Tissues. Toxicon. 47(7): 734-741.

Soares, R.M., M. Yuan, J. C. Servaites, A. Delgado, V. F. Magalhães, E. D. Hilborn, **W. W. Carmichael**, S. M.F.O. Azevedo. 2006. Sub-lethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro – Brazil. Environmental Toxicology. 21 (2): 95-103.

Yuan,M. **W. W. Carmichael**, E. D. Hilborn. 2006. Microcystin Analysis in Human Sera and Liver from Human Fatalities in Caruaru, Brazil 1996. Toxicon. 48:627-640.

Daniel Dietrich

General questions

1. *Are the Toxicological Reviews logical, clear and concise?*

The reviews for Anatoxin A and cylindrospermopsin are clear and concise, although in some cases there are still mistakes in the documents as well as missing information that should be considered. With regard to the microcystin review, most of the data is clear and concise, HOWEVER, the data doesn't make a clear data quality distinction between data obtained with pure toxin, well characterized extract or bloom material, and more or less non-described /anecdotal bloom data. Moreover, the section on ADME appears rather confusing, integration of tables from more recent reviews[1], could have been very helpful. More importantly, the microcystin reviews do NOT describe the actual data for human exposure to algal supplements (*A. flos-aquae*) and neither consider the risk associated with these exposures into any of the risk calculations. Similarly, the cancer risk calculations, largely underestimate the potential cancer risk associated with tumor promotion despite that the reviews does cite and evaluate the associated data set. More importantly the recent finding and classification of IARC/WHO in classifying MC-LR as a class 2B carcinogen should be taken into consideration and also cited [2].

2. *Has EPA accurately, transparently and objectively represented and synthesized the scientific evidence for non-cancer and cancer hazards?*

Anatoxin-a: For this review the answer to above question is YES, despite that some sections appear incomplete or some interpretations appear weak or not as conclusive as presented (specific comments and literature to the review will be detailed later in this commentary).

Cylindrospermopsin: For this review, the data regarding kidney toxicity (4.5.2) are completely wrong and falsely interpreted (specific comments and literature to the review will be detailed later in this commentary).

Microcystins: For this review the answer to above question is NO. The ADME section appears confusing, the cancer risk section lacks proper evaluation of the human exposure and health risks associated with voluntary intake of *A. flos-aquae* algal supplements, and the cancer risk evaluation does not consider tumor promotion with the appropriate risk factors nor the present evaluation by IARC/WHO ([2]).

3. *Are you aware of additional studies that should be considered in the assessment of the non-cancer and cancer health effects of these toxins?*

Anatoxin-a: [3-13]

Cylindrospermopsin: [14-22]

Microcystins: [1; 2; 23-28]. A subchronic initiation-promotion study with aged rats by Michel Charbonneau et al in Montreal supports a NOEL of 40 µg/kg day, whereby effects assessed were increased numbers and sizes of preneoplastic GST positive liver foci.

4. *Please discuss research that you think would be likely to reduce uncertainties in the reference values for future assessments.*

Anatoxin-a: Actual exposure data for humans incl. some kinetic evaluations with radio-labeled Anatoxin-a as well as a better comparison to effects of nicotin in humans would allow to refine the risk assessment. That said it is important also to review the current indications that consumption of algal supplements incl. Spirulina may contain copious concentrations of anatoxin-a[11].

Cylindrospermopsin (CYN): A proper 2-year study with cylindrospermopsin would allow improving the non-cancer and cancer risk assessment. Proper evaluation for the current kinetic data demonstrates that contrary to current interpretations[14] it does appear that CYN is taken up via organic anion transporting proteins (OATP). A 42-week study with male and female mice was conducted by Sukenik et al.[19]. These authors used oral exposure (drinking water) and doses between 100 to 500 µg CYN/L (corresponding to 10-55 µg CYN/kg bw day). They determined a LOEL of 20µg/kg bw day and a NOEL of 10 µg/kg bw day.
For an acute exposure the study by Stewart et al.[21] should be considered, which demonstrated an increased irritant and cutaneous sensitizing properties of CYN in mice.

Microcystins: As the principle risk assessment of MC-LR relies solely on the Heinze et al 1999 study which lacks multiple doses and especially doses in the low dose range and lacks highly reliable pathological evaluation of the liver histopathology, a repeat subchronic study with rats and mice of both sexes would be advisable and also feasible. Similarly, the tumor promotion by MC-LR and other congeners should be re-addressed in a fashion that will allow more reliable incorporation oft eh data into cancer risk assessment.

Anatoxin-a
DETAILED COMMENTS:

Page 3, Line 25: Data from Sigma (Sigma 2006 MSDS a224dat.pdf) demonstrates data for the +-enantiomer

Page 3, Table 2-1: Molecular weight for + anatoxin-a Hydrochloride is wrong, it should be **201.69**

Page 6, Line 30-34: The study by Mahmood et al 1988[12]) and the analytical techniques by Gugger et al (2005) and James et al (2005)[9; 10] should be taken into consideration and evaluated in the context of the toxicology studies.

Page 7, Line 10-30: Increasing the number of parameters evaluated will decrease the statistical power of being able to detect real significant changes, while increasing the probability of a type I error (of wrongly concluding that a significant effect occurred where there was none). Bonferroni adjustment, while reducing the type I error would reduce the power to such an extent that only the most overt effects of anatoxin-a would be observed (see also[29]). I would strongly suggest that a statistician would be looking at the data by Fawell and James, 1994 and Fawell et al., 1999, prior to making a decision whether or not to use these studies as principle studies for a short-term RfD (see below).

Page 10 Lines 1-7: If doubt is raised with regard to the data and the author (McPhail of EPA) can be contacted, this should be done in order to reduce uncertainty in the data.

Page 12, Lines 31-34: If hydrocephaly occurred even in the controls, the question needs to be answered whether or not this also occurs frequently in these hamsters (historical controls) before it can be concluded that this has nothing to do with the exposure.

Page 15, 4.5.3 Other Studies: It should be noted that the ROS as specified in these studies appear to be unspecific as rather high doses were employed.

Page 18, Lines 33-45: Specific tests to assess nicotinic activity in order to address anatoxin-a interaction with the nicotinic receptor were not conducted. This should be taken into consideration when evaluating the data with regard to determining a NOAEL.

Page 23, 5.2.3 Short-Term Duration: Due to the high number of parameters evaluated and the rather crude endpoints, I question whether any effects could have been observed at doses <2.5 mg/kg. Indeed, no nicotinic receptor endpoints were evaluated, while lasting toxicologically significant effects would not be expected for anatoxin-a in the surviving animals.

1. *The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute and chronic exposure durations.*

Do you agree with this conclusion?

YES, especially when considering the points raised above.

Is the rationale for not developing acute or chronic oral RfDs transparent and objective?

Not quite! Indeed, the rationale is primarily based on the anecdotal human death "associated" but never proven or properly evaluated with the exposure to anatoxin-a. Due to the severe limitations of this study (Behm 2003, and Carmichael et al, 2004) they should not be mentioned as part of the rationale.

2. *The 28-day gavage study in mice (Fawell and James, 1994; Fawell et al., 1999) was selected as the basic for the short-term oral RfD.*

Is the selection of this study as the principal study appropriate?

YES; although better-designed new studies would be necessary to reduce uncertainty.

Is the rationale for selecting this study transparent and objective?

YES

3. *In the 28-day gavage study selected as the basis for the short-term RfD, two potentially treatment-related deaths were reported. The original study authors concluded that NOAEL for this study was 0.1 mg/kg-day based on these two deaths. This conclusion was based on their inability to determine the cause of death (i.e., to completely rule out a relationship with treatment), and they indicated that the true NOAEL may actually be 2.5 mg/kg-day.*

Due to the low incidences of mortality (that showed no dose-response or gender consistency), the lack of characteristic clinical signs of acute neurotoxicity in the two animals that dies, and the absence of toxicologically significant effects in the surviving mice, as well as the lack of effects at 2.5 mg/kg-day in mice reported in 5-day and developmental toxicity studies (Fawell and James, 1994; Fawell et al., 1999), EPA concluded that the deaths are likely to be incidental and that the actual NOAEL is 2.5 mg/kg-day.

Is the designation of 2.5 mg/kg-day as the NOAEL for this study scientifically justified?

YES, HOWEVER, if at any time better-designed studies with more defined endpoints are available this NOAEL should be reevaluated and the RfD revisited.

Has the rationale for this designation been transparently and objectively described?

YES

4. *The 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980) was selected as the basis for the subchronic oral RfD.*

Is the selection of this study as the principal study appropriate?

In the absence of better studies, YES. HOWEVER, if at any time better-designed studies with more defined endpoints are available this NOAEL should be reevaluated and the RfD revisited.

Is the rationale for selecting this study transparent and objective?

YES

5. *Are the uncertainty factors applied to the points of departure (PODs) for the derivation of the short-term and subchronic RfD values scientifically justified and objectively described?*

YES

Cylindrospermopsin

DETAILED COMMENTS:

Page 3. Line 21: Stereocenters NOT ster*i*ocenters

Page 12 paragraphs 2 and 3 and SECTION 4.5.2: The interpretation by the original authors as well as by the review team appears wrong! As CYN can be taken up by OATPs it would be expected that CYN induces tissue damage at lower concentrations in the liver than in the kidney (as also observed by Humpage and Falconer, 2003). As mice were used, serum albumin is not a very good measure of decreased hepatic protein synthesis. On the contrary, the authors should have looked for mouse urinary protein (a protein excreted by male mice at approx. 15 mg/ 24 hrs, i.e. 10-16 mg/24 hrs and a urinary excretion of 18.2-29 mg/day/g kidney with kidneys of 0.55 g weight, see also [15-18]). Mouse urinary protein is synthesized specifically in the liver of male mice and excreted via the urine, thus any reduction in urinary protein will be testimony of a potential liver damage or decreased protein synthesis. Moreover, the increased kidney weight is most likely a compensatory reaction to the low amount or urinary protein in the primary urine due to limited protein synthesis in the liver.

Page 16, Lines 27-28: Statements such as “it has generally been considered” should be avoided as no proof of principle has been forwarded in the literature so far.

Page 17, 1st paragraph: Again no real proof of inhibition is given by Terao et al, 1994. Indeed, the data provided could just as well be interpreted as being characteristic of a peroxisomal proliferation and increased peroxisomal function.

Page 18-19, Section 4.5.2: See comments made to page 12. Indeed, mouse urinary protein is the major urinary protein in male mice! Thus the last paragraph doesn't make any sense and is in complete disagreement with the physiological understanding of the male mouse CYN exposure data. Moreover, the male mouse data regarding renal toxicology cannot be readily extrapolated to humans and other mammalian species as the male mouse urinary proteins were certainly not THP! I strongly suggest a complete revision of this section. As no female mice were exposed, the male mice data cannot be readily used.

Page 19-20 Lines 43-2: These findings could very well explain the observed renal pathology at higher CYN doses as well as the lack of visible liver pathology in the male mice exposed to very low doses of CYN.

Page 20, Lines 4-12: The publication by Reisner et al.[22], regarding the inhibition of the uridine monophosphate synthase complex by low doses of CYN should be reviewed here. Moreover, this paragraph serves well to explain the reduced protein synthesis as observed in the male mice orally exposed to CYN (Humpage and Falconer, 2003).

Page 21, Lines 8-11: I would not consider the clinical picture as well defined. Most if not all cyanobacterial intoxication will present with a similar clinical picture. Similar clinical pictures will also be observed following mushroom intoxications (e.g. death caps).

Page 21, Lines 17-19: The current data does not allow stating that renal effects follow a dose-severity progression. Both the male mouse (MUP) and the male rat (□2u-globulin) are the wrong models to allow detection of decrease hepatic protein synthesis via urinary protein analysis and derivation of renal toxicity.

Page 23, 4.6.3 Mode of Action: I think this section needs a complete revision as 2 modes of action (apparently also governed by dose) appear to preside:

a.) Specific cytotoxicity and genotoxicity
b.) Protein synthesis inhibition, peroxisomal proliferation/ increased activity

Page 27, 5.2.4.1: Choice of principal study is hampered by the fact that the interpretation of the study is severely questionable based on lack of knowledge of male mouse physiology. Moreover, it is possible that the POD is wrong due to the inadequate animal model.

Page 28, Line 28: The BMD of 43.1 µg/kg-day stated does not agree with the BMD 43.9 µg/kg-day tabulated in table 5.2.

1. *The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute or short-term exposure durations*.

Is the selection of this study as the principal study appropriate?

In the absence of better studies, YES. HOWEVER, if at any time better-designed studies with more defined endpoints are available this should be reevaluated.

Is the rationale for selecting this study transparent and objective?

YES

2. *The 11-week gavage study in mice (Humpage and Falconer, 2003) was selected as the basis for the subchronic oral RfD.*

Is the selection of this study as the principle study appropriate?

NO, due to the restrictions and limitations in the interpretation of the study as stated above.

Is the rationale for selecting this study transparent and objective?

YES

3. *The critical effect identified in Humpage and Falconer (2003), was increased relative kidney weight.*

Is selection of this finding as a critical effect scientifically justified?

With severe limitations, as stated above see comments to pages 12, 18-19, 21 and 23.

Is the rationale for selecting this effect transparent and objective?

NO, wrong premise at the outset and wrong data interpretation-

4. *Benchmark dose (BMD) modeling was utilized to estimate a BMD and BMDL for increased mean relative kidney weight using 1 standard deviation above the control mean as the benchmark response level (BMR). After dropping the high dose group, the linear model adequately fit the data and produced as estimated BMD of 43.1 mg/kg-day and BMDL of 33.1 mg/kg-day.*

NOTE the discrepancy between the text BMD of 43.1 mg/kg-day and the tabulated BMD of 43.9 mg/kg-day!

Was the modeling appropriately conducted and interpreted?

YES, with limitations to the numbers employed.

The BMDL of 33.1 mg/kg-day was chosen as the POD of RfD derivation. Is the rationale for selecting this as the POD transparent and objective?

YES with limitations due to the questionable validity of the male mouse as the model (see comments to pages 12, 18-19, 21 and 23.).

5. *The conclusion was reached, that the 11-week gavage study in mice (Humpage and Falconer, 2003) selected as the basis for the subchronic oral RfD study could not be utilized for the derivation of a chronic oral RfD due to the excessive uncertainty inherent in doing so.*

Do you agree with this conclusion?

YES entirely, see also comments to pages 12, 18-19, 21 and 23.

Is the rationale for not developing a chronic oral RfD transparent and objective?

YES

***6.** Are the uncertainty factors applied to the point of departure for the derivation of the subchronic oral RfD scientifically justified and transparently and objectively described?*

YES, although when reviewing the applicability of the male mouse as surrogate model for the human this may need to be revisited, especially as the male mouse data may not be readily extrapolated to the human and therefore larger uncertainty factors may be necessary.

Microcystins (LR, RR, YR and LA)

DETAILED COMMENTS:

Page 3, Lines 26-30: The Special Issue on Cyanobacterial Toxins in TAAP 203(3) 2005 should be taken into consideration for gaining additional data. In addition, more physico-chemical data can be obtained from the PhD thesis by Lisa Spoof, Turku University Finland [30].

Page 5, Chapter3. Toxicokinetics: A nearly complete evaluation of ADME was conducted by Dietrich and Hoeger [1]. The data were assembled in one table that allows rapid overview of the most important data. It appears helpful to include table 1 from Dietrich and Hoeger 2005 into the chapter 3 presented here.

Page 6, Section, 3.2, Lines 21-23: Although in principle correct, there is limited data to really prove that microcystins need active transport. This evidence is clearly limited a but a few of the >80 congeners known to date. I thus would suggest to revise this generalized statement.

Page 6, Section 3.2.1: This section appears incomplete and incorrect. Please compare with Dietrich and Hoeger 2005 and then revise.

Page 7, Lines 1-5: This paragraph citing Brooks and Codd (1987) is meaningless especially as the presence of microcystin nor the type of microcystin congener was not confirmed.

Page 7, 2nd paragraph: Although Robinson et al 1989 is cited, the i.v. study with mice [31] by the same authors is not. The latter study however, is just as important as the i.p. study.

Page 7, 4th paragraph: Although the study by Ito et al 1997a is interesting it has little bearing on actual tissue distribution, as the study is hampered by recognition of covalently bound microcystins in the various tissues as well as the detection limit of the microcystin antibody in the immunohistochemical assays employed.

Page 8, Lines 2-4: The question is whether or not the microcystins would have been detectable in the milk if they had been present. Indeed, the methods used were rather insensitive and thus question the interpretation.

Page 8, Lines 27-34: This section appears incomplete and incorrect. Reference is missing to Fischer et al. 2005[26]. The xenopus oocytes were made to express human OATP as well as rat OATP and NTCP. The microcystin used was 3H-dehydro-microcystin-LR. Reference to section 4.4.5.1 appears inconsistent with the text of this very paragraph.

Page 8-9 Lines 43-9: This paragraph appears incorrect and outdated. Runnegar et al 1995b should be consulted to assure that citation and context was correct.

Page 9, 2nd paragraph: Toivola et al. [32-35] as well as Batista et al [36] should be incorporated into this paragraph.

Page 9, Section 3.2.3. This section should be revised and expanded including additional publications[37-45].

Page 10, 3rd paragraph: As dihydroMC-LR cannot covalently bind to cystein groups, i.e. neither the Cys273 of PP1 nor the Cys266 of PP2A, how can Hooser et al find 50% covalent binding to proteins? This appears to be contradictory to all present knowledge and thus should be placed into a more critical context.

Page 10, Lines 27-35: There are a number of ELISAs available. The ELISA used here should be specified as well as critically evaluated with regard to limit of detection. The MMPB method is NOT beyond doubt as Lemieux oxidation thus far has not unequivocally demonstrated to produce mass fragments solely attributable to microcystins.

Page 10-11, Metabolism: This section is rather iterative than a real summary of the present weight of evidence. A complete revision is advisable.

Page 15, 4 Hazard Identification: This chapter is missing any description of children and adults potentially exposed to microcystins via alga supplements based on *A. flos-aquae*. This is a serious deficiency in the document from the review stand-point as well as for the risk assessment conducted later in this review. It is a MUST to include as a minimum the following publications: [1,46-47] and references cited therein. Moreover, the human case studies in Sweden[27] and Finland[28] should be included.

Page 16, Lines 1-9: The presence of LPS was not ruled out but was not determined either. However as LPS produces similar clinical symptoms as the ones described here, LPS as a possibility must be mentioned.

Page 16, 2nd paragraph: This paragraph describes the human fatalities in Itaparica Brazil. Unfortunately this is not well described and sometimes incorrect. Please refer back to the original paper or consult with Dietrich and Hoeger 2005 for correct presentation of facts.

Page 19-24, Section 4.1.1.2.: This data was extensively evaluated by the IARC WHO[2]. The respective IARC monograph could be employed to add onto the data presented in this section.

Page 24, Section 4.1.2 Inhalation exposure: At least anecdotal exposure data are available from Finland[28].

Page 31, 2nd paragraph: The exposure of the mice was 43 days, HOWEVER, the microcystin concentration in the algal supplements fed to the mice was never ascertained but rather extrapolated from historical data. Consequently the data by Schaeffer et al 1999 should be viewed with extreme caution.

Page 34, 4.2.1.3.2 Cyanobacterial extracts: This is a pivotal study and thus should be presented in much more detail. More importantly is the fact that a LOAEL but no NOAEL was determined.

Page 43, Line 3: Concentrations of Microcystins should either be given as MC-LR equivalents or then as sum of all congeners whereby each congener was quantified, but NOT as “microcystins/L”.

Page 55, 4.4.7.1 Target Organ/Cell Specificity: This section should include the pivotal study by Fischer et al {Fischer, 2005 #59. More importantly organ-blood perfusion should be specifically noted when organ specificity of microcystin accumulation/toxicity is discussed.

Page 58, last paragraph: Eriksson et al 1990b did NOT use MC-LH. They used MC-LR, -RR and 7-dmMC-RR.

Page 62, 1st paragraph: The ATP-synthase inhibiting capacity of microcystin-LR {Mikhailov, 2003 #5812} should be noted here was well.

Page 70, 4.5.1: Blood perfusion of the different organs should be noted and placed into context with the purported target organ specificity of microcystins.

Page 74, 4.6 Synthesis: Reference to Annadotter et al 2001 [27] and Hoppu et al 2003 [28] is missing. All reference and evaluation of human exposure to microcystins via algal supplements are missing [1,46-47].

Page 81, Section 4.8: All reference and evaluation of human exposure to microcystins via algal supplements are missing [1,46-47].

Page 95, 5.4 Cancer Assessment: Although the statement is correct there is sufficient tumor promotion data that would allow deduction of a cancer risk (see IARC/WHO, monograph and [2])

Page 6.2 Dose response: The tumor promoting capacity of microcystin-LR should be taken into consideration and included in the UF, possibly via an additional UF of 3.

1. *The conclusion was reached that the available toxicity information was inadequate to support derivation of oral RfD values for microcystin-RR, -YR or LA.*

Do you agree with this conclusion?

YES

Is the rationale for not detecting oral RfD values transparent and objective?

YES

Microcystin-LR

1. *The conclusion was reached that the available oral toxicity information was inadequate to support derivation of an acute oral RfD value.*

Do you agree with this conclusion?

YES

Is the rationale for not detecting an acute oral RfD transparent and objective?

YES

2. *The 28-day drinking water study in rats (Heinze, 1999) was selected as the basis for the short-term and subchronic oral RfDs.*

Is the selection of this study as the principal study appropriate?

YES but only in view of the lack of any better studies. This study is inadequate due to the limited number of doses and the rather mediocre histopathological assessment. If better and more well-designed studies become available the RfD should be revisited and a new POD defined.

Is the rationale for selecting this study transparent and objective?

YES

3. *Liver toxicity observed by Heinze (1999) included liver lesions, serum enzyme changes and changes in relative liver weight. All three of these endpoints were considered for determining the point of departure for RfD derivation. BMD modeling was utilized to estimate a BMD and BMDL for each of these endpoints.*

See comments above.

Was the modeling appropriate conducted and transparently and objectively presented?

YES

For the purposes of BMD modeling, the moderate and severe liver lesion categories reported by Heinze (1999) were combined and the BMD and BMDLs for these lesions estimated by the log probit model were 11.0 and 6.4 mg/kg-day, respectively. The BMDL of 6.4 mg/kg-day was chosen as the POD for RfD derivation.

Is the rationale for selecting this as the POD transparent and objective?

YES

Is selection of liver lesions as a critical effect scientifically justified? Is combining the moderate and severe lesions scientifically justified?

YES, however only in view of the lack of a better study.

Is the rationale for selecting this effect transparent and objective?

YES

4. *Data from the 90-day gavage study in mice conducted by Fawell et al. (1999) was considered for the derivation of the subchronic RfD. The BMDL from the Heinze (1999) study (6 mg/kg-day) is approximately an order of magnitude lower than any of the BMDL values derived from endpoints from the 90-day gavage study (57-66 mg/kg-day) therefore, the BMDL of 6 mg/kg-day from Heinze (1999) was chosen as the POD for subchronic RfD derivation.*

Do you agree with this decision?

YES

Is the rationale for selecting 6 mg/kg-day as the POD transparent and objective?

YES

5. *The 18-month drinking water study in mice (Ueno et al., 1999) was selected as the basis for the chronic oral RfD. This study used only a single dose level and identified a freestanding NOAEL but was chosen for RfD derivation because it was a well-conducted study of chronic duration and employed a relevant exposure route (drinking water).*

Is the selection of this study as the principal study appropriate?

This is the only study with any animal that uses prolonged oral exposure. As only female mice were used there are some caveats, however as this is the only study the limitations of this study are outweighed by the fact that this is the only chronic study available.

Is the rationale for selecting this study transparent and objective?

YES

6. *Are the uncertainty factors applied to the points of departure for the derivation of the short-term, subchronic and chronic oral RfDs scientifically justified and transparently and objectively described?*

YES, for the acute and subchronic situation.

I would add more UFs to the chronic situation in order to accommodate the cancer risk (tumor promotion potential). Otherwise the UFs are well defined and explained.

Questions related to the cancer assessment for anatoxin-a, cylindrospermopsin and microcystins (LR, RR, YR and LA)

1. *Do the available data support the conclusion that the database for each of these toxins provides inadequate information to assess carcinogenic potential based on the weight-of-evidence categories in the EPA 2005 Guidelines for Carcinogen Risk Assessment?*

I would agree with regard to the cancer risk of Anatoxin-a, Cylindrospermopsin, Microcystins-LA, RR, and YR. For Microcystin-LR there are ecological studies as well as animal tumor-promotion studies that would provide sufficient evidence for carcinogenic potential. Moreover, the carcinogenic potential of MC-LR was also evaluated by IARC/WHO and it was concluded that there is sufficient evidence to classify MC-LR as a 2B carcinogen [2].

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45. **Mikhailov, A., Harmala-Brasken, A.S., Hellman, J., Meriluoto, J. and Eriksson, J.E.,** Identification of ATP-synthase as a novel intracellular target for microcystin-LR, *Chemico-Biological Interactions*, 142(3), 223, 2003.
46. **Gilroy, D. and Chu, F.,** Deriving a safe level for microcystin toxin in blue-green algae dietary supplements (abstract), *Toxicological Sciences*, 42(1-S), 227, 1998.
47. **Gilroy, D.J., Kauffman, K.W., Hall, R.A., Huang, X. and Chu, F.S.,** Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements, *Environmental Health Perspectives*, 108(5), 435, 2000.

Maria Runnegar

Toxicological Reviews of Cyanobacterial Toxins: Anatoxin a
Comments
General
The anatoxin a review is a clear summary of the field. The information is without detectable bias.
No I am not aware of other suitable studies that could be considered for assessment.
As with the other cyanobacterial toxins conventional toxicity studies need to be done in at least two species that include metabolic and behavioral end-points. This could lead to a decrease in uncertainties in the reference values. Because of the mode of action of the toxin as an agonist at the neuromuscular nicotinic receptors it is unlikely to have carcinogenic potential particularly when other agonists are considered.

Specific questions.
There is no way to establish oral RfD values for chronic toxicity as, as far as this reviewer is aware, no studies have been carried out. The study of Stevens and Krieger (1991) could potentially have been used to establish acute oral RfD. The authors state that six mice were used for each dose to establish the LD_{50} of 16.2 mg/kg (CI 15.4-17.0). Unfortunately the authors present no other information for this treatment group.

The literature has essentially only two studies that address the short term and subchronic oral toxicity of anatoxin a: those of Fawell et al. 1999 and Astrachan et al. 1980, Astrachan and Archer 1981. Neither of these two studies is ideal, but both arrive at values that are at least qualitatively consistent.

The selection of Fawell (1999) is appropriate for the determining the short term oral RfD. The assessment of the study in the review is transparent and objective.

I agree with EPA that the two mouse deaths in Fawell (1999) 28 day gavage study are on balance not related to anatoxin a toxicity. It is the experience of this reviewer that the response to cyanobacterial neurotoxins in rodents is essentially an all or none effect and that the symptoms and the short time to death after dosing are very consistent and reproducible.

The seven-week drinking study in rats (Astrachan, 1980, Astrachan and Archer 1981) is just about long enough in duration to qualify as a subchronic study. Under ideal conditions rats (not calorie restricted) will live on average close to three years (data from NIH). Even in a less ideal environment rats would be expected to live at least two years if free of disease. The selection of this study is therefore appropriate and its choice transparent and objective

Given the very limited data from the two studies it is prudent to have such uncertainty factors. With more information the uncertainty factors will most likely be decreased given the mode of action of anatoxin a.

Toxicological Reviews of Cyanobacterial Toxins: Cylindrospermopsin

Overall general comments
This review is a very well prepared, comprehensive review of the literature and studies for these cylindrospermopsin . It is well written, the content is clear. I can only speak from the perspective of being familiar with the field, but I believe that a reasonably educated and motivated person would be capable of understanding the report.

There are some additional studies that should be included for completeness but on the whole these do not add significantly to the assessments.

I am not aware of research that would likely reduce uncertainties in the reference value for future assessments

A minor point: would it be of value to report for the ratio between oral and IP dosage explicitly?

1. Acute and short-term exposures
There are numerous reports of dose dependent animal death following intraperitoneal dosing and some for oral dosing. The nature of the hepatic lesions following cylindrospermopsin intoxication have been studied and reported in detail. Because the aims of that work were and are different from what is needed here, at best these reports can only be used to confirm, reinforce the RfD derived from the subchronic oral study of Humpage and Falconer (2003).

Subchronic oral dosing.
The study of Humpage and Falconer (2003) remains the only comprehensive study of the effects of oral dosing of cylindrospermopsin over a period of 11 weeks in mice. Eleven weeks represent about 10 percent of the expected lifespan of mice therefore the study satisfies the criteria of a subchronic study.

3 Increased relative kidney weight.
In Humpage and Falconer (2003) the kidney weight of mice was significantly increased at a gavage dose of cylindrospermopsin of 60 µg/kg. The authors postulate that this may a compensatory change because of protein synthesis impairment. This has not been demonstrated as the extent of protein inhibition was not addressed in the study. Nevertheless it is prudent here to take this kidney weight change as reflecting cylindrospermopsin toxicity.
In agreement with the Falconer Humpage (2003) study, an increase in kidney weight has been recently shown to occur in mice dosed orally with *Aphanizomenon* extract containing cylindrospermopsin (dose 10-55 µg/kg) over a period of 20 and 42 weeks (Sukenik et al Environ Toxicol 21, 575 2006, Table 2)

4. The benchmark dose modeling was appropriately done

5. Yes, it would be wrong at this stage to assume that the changes seen at 11 weeks will be identical to those seen in a future chronic dosing study. This is particularly so because of the lack of pharmacokinetic data, the possibility of carcinogenic activity as well the additionally findings described by Sukenik et al (2006)

The uncertainty factors are scientifically justified given the small number of studies that are available.

Specific comments

Page 2 line 9
Humpage and Falconer 2003 do not discuss the chemical structural aspects of cylindrospermopsin

page 4
in Norris et al 2001 large standard deviations are shown for the findings

page 5
Metabolism
If, as quoted in Norris et al 2002 (Seawright personal communication to Norris), an inbred strain of mice (C57/B16) could not be protected by P450 inhibition, extrapolations to other species require caution till more detailed studies of the role of P450 enzymes in cylindrospermopsin in vivo toxicity are done

Page 7
As stated in the comment by Ian Falconer the symptoms of the Palm Island outbreak are not consistent with copper poisoning
Palm Island poisoning. Section 4.1 lines 13,28.
"The individuals poisoned initially showed constipation, and only 2-3 days later had profuse bloody diarrhea. This rules out copper poisoning which is immediately followed by diarrhea. This event is discussed in (my) book pages 88-90"

page 8
The lack of dose response in Pilotto et al (2004) does not allow the conclusion that cylindrospermopsin is a skin irritant

Page 9
lines 1-18
it is not quite correct that cylindrospermopsin concentration cannot be determined in cell extracts or supernatants (as an example see Sukenik et al 2006). It simply requires assay of the compound and then it can be expressed as cylindrospermopsin equivalents. It is typical of most if not all cyanobacterial secondary metabolites to be synthesized and released in very different amounts depending on stage of growth and conditions
lines 18-23
the difference in LD_{50} between Hawkins et al (1997) and Ohtani et al (1992) is decreased significantly when the 5-7 day LD_{50} are considered. The tenfold difference at 24 hrs in the calculated LD_{50} at 24 hrs was lost by 5-7 days when the calculated cyanobacterial equivalent was 36 mg/kg of extract for Ohtani et al and 32 mg/kg extract for Hawkins et al (1997).
It is possible that the cell extracts might have contained other toxins but this was not shown in this study. Different strains of mice were used in the two studies. See also Falconer and Humpage 2001 for LD_{50} values ip in the mouse

Page 12
Subchronic studies
4.2.1.3.2. Cell Extract Studies
In Sukenik et al (2006) mice were exposed to cylindrospermopsin containing cultures of *Aphanizomenon ovalisporum* for 20 and 42 weeks. This study was for a longer period of exposure than for conventional subchronic toxicity studies, but it was not a whole life study.
The cylindrospermopsin concentration in the water was gradually increased from 10 to 55 µg/kg (cylindrospermopsin equivalents). As in previous studies liver weight and kidney weight increases were shown in the treated mice. The increase in liver weight was slight when compared to the percentage increase in kidney weight. The authors propose 20 µg/kg/day as the LOAEL. What differs from previous work is the report of the presence of acanthocytes (deformed red cells) accompanying hematocrit increases in the treated mice. These changes were already seen at 20 weeks. No effect on red cell structure has been reported in other work. Two possibilities may explain this. Humpage and Falconer (2003) dosed mice with purified (0-240 µg/kg/day) cylindrospermopsin for a period of 11 weeks and with extract containing cylindrospermopsin (cylindrospermopsin equivalents 0 to 657 µg/kg/day) for 10 weeks. An hematological examination of the mice was part of the protocol. No significant hematological abnormalities were reported. It is conceivable therefore that the red cell lesions only occur after longer

exposure to cylindrospermopsin. Another explanation could be that Sukenik et al (2006) used cultures of *Aphanizomenon* rather than of *Cylindrospermopsis*. It is therefore possible that the erythrocyte changes result from other component(s) that were present in this cultures. These other components could affect red blood cells independently of cylindrospermopsin or perhaps acting synergistically with the toxin.

Page 13
Section 4.3
Ian Falconer reported that Dr Neil Chernoff (EPA, Research Triangle) has recently completed studies on the teratogenicity of cylindrospermopsin. This reviewer does not know what were the findings of this study. If known these need to be included.

Page 14
Other Studies
4.4.1.1. Studies of purified cylindrospermopsin**.**
Line 11
The purity of the cylindrospermopsis was not reported but this preparation was used to determine the chemical structure of cylindrospermopsin. It therefore would be unlikely to contain significant impurities (Ohtani et al 1992).
Line 15 Add
"and sacrificed at intervals between 16 and 100 hours after dosing."

Page 16
Genotoxicity
Line 18. Add
" These authors suggested that the opposed findings in the studies could be reconciled if a metabolite of cylindrospermopsin (through P450 activation) rather than cylindrospermopsin itself might be responsible for the genotoxic changes described."

Page 17
Liver toxicity
Line 37 Insert
Humpage et al (2005) showed that P450 metabolism of cylindrospermopsin was necessary for its genotoxicity as measured by the COMET assay. Inhibitors of P450 SKF525A and omeprazole decreased the tail moment of mouse hepatocyte nuclei to control levels. The P450 inhibitors did not protect hepatocytes from protein synthesis inhibition by cylindrospermopsin indicating that this inhibition is caused by the natural toxin rather than a metabolite (Froscio et al 2003).

Page 20
Line 1 What does "Is a primary effect of the chemical" mean?
By primary is it meant to be a direct effect of cylindrospermopsin requiring no P450 activation or does it mean that it is not a secondary consequence of the toxicity?
Line 8 Insert
'The protein level and the enzyme activity of tissue transglutaminase in mouse liver were not measured

line 21 change might to is
The sulfate is also not required for the decrease in GSH levels in intact rat hepatocytes Also addition of the diol to hepatocytes resulted in cell death at similar concentrations and times as the natural and the chemically synthesized cylindrospermopsin

Line 43
Natural and chemically synthesized deoxycylindrospermopsin were shown to inhibit protein synthesis in rat hepatocytes with potency similar to that of natural cylindrospermopsin (Looper et al 2005)

Page 24
From Ian Falconer
"page 24 lines 10-12 and 22-23 This is misleading. In the preliminary carcinogenicity study no controls showed neoplasia, whereas 5 of the cyn treated mice had neoplasms. The relative risk of 6.2 was not significant due to limited numbers of animals. (See Falconer 2005 page 105). The author agrees that no conclusion can currently be drawn on the carcinogenicity of cyn, but there is very suggestive mechanistic evidence, and this preliminary study, pointing towards carcinogenicity."
Line 29
Humpage et al (2005) showed that cylindrospermopsin in mouse hepatocytes was genotoxic as measured by the COMET moment assay.

Page 25
Insert any information on teratogenicity

References to insert
Sukenik, A., M.Reisner, S. Carmeli and M.Werman. 2006. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in mice: long-term exposure to low doses. Environ. Toxicol.21: 575-582.

Looper, E., M.Runnegar and R. Williams. 2005. Synthesis of the putative structure of 7-deoxycylindrospermopsin:C7 oxygenation is not required for inhibition of protein synthesis. Angewandte Chemie 44: 3879-3881

'Cyanobacterial toxins of drinking water supplies;
cylindrospermopsins and microcystins' Falconer , Ian R., CRC Press,
2005.pp.279

Toxicological Reviews of Cyanobacterial Toxins: Microcystin-LR, RR, YR and LA
Comments
General
Overall general comments
The review is a very well prepared, comprehensive review of the literature and studies for this cyanobacterial toxin. It is well written, the content is clear. I can only speak from the perspective of being familiar with the field, but I believe that a reasonably educated and motivated person would be capable of understanding the report.

There is an additional study (Falconer et al 1994) that should be included for completeness as corroborating evidence for the assessments.

I am not aware of research that would likely reduce uncertainties in the reference value for future assessments

Not all abstracts in the reference lists and in the text are indicated as such. Most often these abstracts are not peer reviewed and therefore their findings and conclusions are doubtful if not followed or accompanied by a refereed paper. Conversely when there is such a publication then it should be cited in preference to the abstract.

It is unfortunate that for the microcystins as well as for the other cyanobacterial toxins being considered there is a paucity of suitable studies that can be used for the quantitative dose response assessment of the compound. There a number of reasons why this is so.
The cost of purified toxins is a considerable obstacle to such studies.
Up to now there have not been easily identifiable sources of funds or institutional interest for such toxicity studies in the US. In fact the studies used in this report to derive RfD (Heinze, 1999; Fawell, 1999 and Ueno, 1999) have all been carried out outside the US. It is the experience of this reviewer that NIH and NIEHS, given their mission, have chosen to fund studies that address the mechanism of toxicity. There also significant studies using field samples to determine toxicity. The premise for this approach is that animal or human exposure is not to the purified toxins but rather to cyanobacteria and their products in water. Although these latter studies with contaminated water /cyanobacterial extract cannot be used here to reach quantitative values here they reinforce work with the purified toxins in so far as the toxic consequences of exposure are at least consistent when not identical in the two cases.

It has become clear that the expression of toxicity is influenced by the host as much as by the compound. As an example we can consider acetaminophen (work that I am familiar with). Its acute hepatotoxicity has long been known and characterized: activation to NAPQI by cytochrome P450 leading to GSH depletion and the formation of protein adducts. In recent work it has also become clear that the innate immunity status, as well downstream signaling by JNK and other kinases influence the toxicity of the drug.
(see: Liu ZX. Kaplowitz N. Role of innate immunity in acetaminophen-induced hepatotoxicity. *Expert Opinion On Drug Metabolism & Toxicology. 2(4):493-503, 2006*
Gunawan BK. Liu ZX. Han D. Hanawa N. Gaarde WA. Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity *Gastroenterology. 131(1):165-78, 2006*
Liu ZX. Han D. Gunawan B. Kaplowitz N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity *Hepatology. 43(6):1220-30, 2006)*
This would lead to increased uncertainty in the toxic response of a particular individual to any compound as we can expect polymorphism and differential status in these responses as well as in the better characterized P450 and GSH transferases.

Since I have no formal training in toxicology and no personal experience in benchmark dose modeling I can only comment qualitatively about my assessment of the data.

1. I agree that the acute oral RfD value could not be determined. Many of the published studies in which animals were dosed with cyanobacterial bloom extracts also lack some information that would have enabled them to aid qualitatively in the establishment of the acute oral RfD following purified microcystin dosing

2. Short term oral RfDs.
Again data are limited. Heinze 1999 determined the effect of microcystin-LR administered in the drinking water to young rats for 28 days. The two dose levels 50 and 150 µg/kg per day resulted in significant increases in hepatic enzymes as well as histological lesions in the liver. The study is validated since there was a dose response in the increasing severity of the toxic changes.

3. The fact that a constant finding in innumerable studies of microcystin or microcystin containing extract toxicity is that they show dose dependent increases in hepatic enzyme levels in the circulation, in histological damage measured by light or electron microscopy as well as increases in liver weight as percentage of total weight provides the justification for, and validates the use of these parameters in the RfD derivation.

Although not a topic in which this reviewer is familiar, the log probit model appears to have been applied correctly. The only comment is that the very steep dose response curve for microcystin toxicity that has been shown in many studies might have led to an overestimation of toxicity.
One comment on the Heinze study is that young rats were used as experimental animals. From the mouse study of Ito et al (1997a) age was shown to be a determinant of toxicity.

4. Subchronic oral RfDs
When comparing the two studies of Heinze and Fawell there are significant differences in experimental design. Different species: one rat the other mouse, dosing continuous in drinking water in one and in the other by gavage. In addition, as said in 3. above, the toxicity of microcystin in the Heinze study may have been overestimated because of the choice of doses in that study. Nevertheless till future studies clarify this difference it is prudent to go for the lower value particularly given the possible of microcystin for tumor promotion.

5. Chronic oral RfD
Ueno et al 1999
There are no other studies addressing the chronic oral toxicity of purified microcystin. The level of microcystin chosen was very low (mean cumulative microcystin-LR intake of 35.5 µg per mouse over 18 months equivalent to about 1.0-1.5 mg/kg). This is equivalent to a mean dose per day per mouse of 0.07 µg (3 µg/kg).

Short-term/subchronic dosing.
A study by Falconer et al (1994) provides qualitative details for comparison.
This study determined the effect of subchronic dosing of pigs with cyanobacterial extract in water for 44 days
The doses were 1312, 796, 280 and 0 µg microcystin equivalents per kg/day
The 280 µg/kg/day dose was taken as the LOAEL since there was minimal detectable toxicity (in 1 of 5 pigs) or 100 µg/kg/day of microcystin-LR equivalents)
Although this study cannot be included in the RfDs determination because the dosing consisted of microcystin containing cyanobacterial extract rather than the pure compound, it has the advantage of having been done in a non-rodent species.

6. Yes, given that single, not completely satisfactory studies are available for calculating the RfD.

Specific comments for the review of microcystins LR, RR, YR and LA.

Page 3
Line 19 amino acid residues is more appropriate than amino acid peptides

Page 5
Lines 12 to 19
Microcystins with intact methyldehydroalanine are capable of forming secondary covalent bonds with PP2Ac (Craig et al, 1996; Runnegar et al, 1995a) as well as with PP1c (Runnegar et al, 1995a). ^{3}H-radiolabeled dihydromicrocystin has been used in many studies of the uptake and distribution of microcystin in animal and cells. This could lead to quantitative differences between the natural microcystins and the derivative. The inability to form covalent bonds would be more significant for longer term experiments rather than for shorter time studies as the original fast inhibitory binding is very tight and has been shown to be kinetically essentially irreversible (Takai et al 1995 and earlier publications by the same author).
Lines 40-41

Ito et al 1997a found significant uptake of microcystin by the liver. There was no immunostaining of the intestine reported in that study. An immunoaffinity column was used to demonstrate uptake of microcystin by the liver.

Page 6
Lines 22-23
The uptake of microcystin in the liver is by the multispecific organic anion transporting polypeptides
Line 35-36
The structure and composition of the microcystin-YM along with those of microcystin-LR, YR, and YA (at the time named cyanoginosins) used in Falconer 1986 and in Runnegar 1986 and in later publications had been fully characterized (see Botes et al 1985).

Page 7
Lines 2-3
In Brooks and Codd (1987) the peptide toxin was microcystin see references to Botes et al (1983) and to Krishnamurthy et al (1986) in Brooks and Codd. The final nomenclature of the "peptide toxin" as microcystin was only agreed later (Carmichael et al 1988)
Line 25-26
Numerous studies show the formation of GSH adducts of microcystin in vitro. These are catalyzed by the GST transferases. It is most likely that metabolism of microcystin may be at least in part through the formation of GSH adducts. The evidence for this *in vivo* is small. The occurrence of GSH-and cysteine - adducts has only been shown in one study (Kondo et al 1996) In this study very low trace amounts were identified by HPLC co-elution of components of mouse and rat liver extracts with the synthesized adducts.
Line 31-32 not in Ito et al (1997a)
Lines 42-44
In contrast with parenteral dosing Nishiwaki et al (1994) showed no liver accumulation in mice dosed orally with microcystin The authors found that less than 2% of the dose was tissue associated. The authors stated that six hours after dosing the intestinal contents accounted for 37% of the dose. This still leaves 60% unaccounted for. [In this publication the amounts of microcystin injected or given orally cannot be correct as reported. The ip dose of microcystin was given as 2.4 mmoles/mouse (2400 mg/mouse. There is no way that this concentration could be used for ip dosing and result in 70% accumulation in the organ. The oral dose in this work was given as 2.1 µmoles/mouse this is 2.1 mg per mouse or about 80 mg/kg. If mmol or µmol in the paper are meant to be mM and µM then the ip microcystin dose would still be about 25 mg/kg. For the oral dosing 2.1 µM results in a very low dose of only about 25 µg/kg.]

Page 8
Line 16
Now known as multispecific organic anion transporting polypeptide. Replace also in later quoting
Line 19
The perfused liver uptake in Hooser et 1991 was only from n=2 and the two livers had quite different kinetics. A better study to quote is that of Pace et al 1991.
Line 29
It should be Runnegar 1995b rather than Runnegar 1995a

Line 31
The labeled microcystin used by Fischer et al 2005 was ^{3}H- dihydromicrocystin-LR

Page 9
Line 5 add

as cell associated radioactivity and assay….
Line 33
Are proteins of molecular weight 40,000 high molecular weight proteins?

Page 11
Lines 17-22 See comment for page 7 lines 25-26

Page 13
Line 24
Ito et al 1994 did not show secretion of MCLR in the mucus of goblet cells rather they showed by histology lesions in the intestinal wall.

Page 37
4.2.2.2 Short Time Studies
(Benson et al 2005) It is possible that the 20% ethanol might have had an effect on the nasal epithelium allowing more uptake of microcystin

page 40
4.4.2. Immunological Effects
lines 39- 44
Shen et al 2003. The doses of cell free extract used in the study were clearly toxic with significant increase in liver weight (table 1 of publication)
" … treated mice almost appeared sick"
"the corresponding livers were swollen and intrahepatic hemorrhages were observed"
It is therefore possible that the immunological effects observed were a consequence of the microcystin toxicity rather than a specific primary immunological effect of the toxin
The statement that the microcystin content of the extract was 79.53% may not be correct since this would mean that three quarters of the extract is microcystin and one quarter solvent. Taken literally it would mean 79 mg of microcystin in 100 µl

Page 48
Line19-20
For Elleman et al 1978 and Falconer et al 1981 the microcystin was later identified as Microcystin-YM (see comment to page 6)

Page 56
Line 2 insert
But a much larger dose (>100 fold the dose for hepatocytes) and longer incubation times were required

Page 57
Lines 19-20 refers to in vivo dosing (hemorrhage) rather than studies with isolated hepatocytes and perhaps should be moved

Page 60
Line 13 remove "substantial" and substitute with detectable
from Gulledge et al (2003b) the IC_{50} for the inhibition of PP activity by these analogues was more than a thousand fold greater when compared with intact microcystin
lines 23-24 add to references Runnegar et al 1995a

page 62
line 7 this remains to be shown in a cell or *in vivo*.

Page 63
Ding 2001
In this work the authors incubated the hepatocytes with 1 µM microcystin. This is a large dose. Runnegar et al (1981, 1985) showed by scanning electron microscopy that changes in rat hepatocytes can be detected already 5 to 10 minutes after addition of microcystin. These changes indicated that cytoskeletal rearrangement was already occurring. In addition in Runnegar (1987) phosphorylase activation (as a result of PP inhibition) was already seen by 2 minutes after the addition of microcystin-YM (0.6 µM). Therefore it is more likely that the oxidative stress is in response to PP inhibition. The authors cite work that showed that okadaic acid, another PP inhibitor chemically different from microcystins, caused oxidative stress. If oxidative stress is taken as a mechanism of toxicity distinct from PP inhibition then it follows that these two chemically very different compounds share two separate primary targets in the cell. This is unlikely.
Ding et al (2001) also observed significant cell lysis (measured by the release of LDH) by 30 minutes. This is in contrast with what has previously been reported (Runnegar et al 1981, Eriksson 1989 and other publications).

Lines 9-11 Microcystin containing cell extract (from *Microcystis aeruginosa* bloom) was used by Ding et al 2000a not the isolated toxin.

Page 66
Line 26 Matsushima-Nishiwaki et al 1995 is not included in the list of references.

Page 72
Line 10 "oxygen species have been shown to precede morphological changes in hepatocytes"
Is this from Ding et al 2001? See above page 63
Line 35 (Runnegar et al 1995b, Figures 1 and 3) 5 µM Cyclosporin A was shown to inhibit the uptake of microcystin in isolated rat hepatocytes therefore the protection could be at least in part due to decreased cellular microcystin

Page 73
Line 32 there are no known compounds that prevent the PP inhibitory activity of microcystin. Experimentally it has been shown that protein phosphatase activity *in vivo* (Runnegar et al 1991) or in hepatocytes (Runnegar et al 1995b) was only protected when uptake of microcystin was inhibited.
Line 35-36 in an earlier study (Runnegar et al 1995b, Figures 1 and 3) 5 µM Cyclosporin A was shown to inhibit the uptake of microcystin in isolated rat hepatocytes

Page 74
Line 27 High acute doses of microcystin have been shown to result in death in laboratory animals

Pages 75
line 45 and continuation of text in line 1 page 79 seem to miss some text. (pages 76-77 table 4-11)

Page 95
Cancer Assessment
There is no significant evidence of direct carcinogenicity of microcystin but there is substantial evidence of tumor promotion. In June 2006 the International Agency for Research on Cancer of WHO in Lyons convened a workshop to evaluate the carcinogenic risks to humans of microcystins and nodularin. The full monograph publication (IARC Monographs Volume 94, June 2006) should be published very soon. A summary of the findings that microcystin-LR is "possibly carcinogenic to humans (group 2B)" has been published (Gross et al 2006).

References to insert

Botes D.P., L. Wessels, H. Kruger, M.T.C. Runnegar, S. Santikarn, R.J. Smith, J.C.J. Barna , D.M. Williams. 1985. Structural studies on cyanoginosins-LR, -YR, -YA, and -YM, peptide toxins from Microcystis aeruginosa. J Chem Soc, Perkin Trans: 2747–2748

Carmichael, W.W., V. Beasley, D. L. Bunner, J.N. Eloff, I. Falconer, P. Gorham, K-I. Harada, T. Krishnamurthy, Yu Min-Juan, R.E. Moore, K. Rinehart, M. Runnegar, O.M. Skulberg,M. Watanabe 1988. Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26(11): 971-973.

Grosse, Y., R. Baan, K. Straif, B. Secretan, F. El Ghissassi, V. Cogliano and on behalf of the WHO International Agency for Research on Cancer Monograph Working Group. (2006). Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet Oncology 7(8): 628-629

Takai, A., K. Sasaki , H. Nagai, G. Mieskes, M. Isobe, K. Isono and T. Yasumoto. 1995. Inhibition of specific binding of okadaic acid to protein phosphatase 2A by microcystin-LR, calyculin-A and tautomycin: method of analysis of interactions of tight-binding ligands with target protein. Biochem J. 306(Pt 3): 657-66.

Dave Stone

Toxicological Reviews of Cyanobacterial Toxins

Anatoxin-a Toxicological Review:

1. The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute and chronic exposure durations. Do you agree with this conclusion? Is the rationale for not developing acute or chronic oral RfDs transparent and objective? If you do not agree with the conclusion that data are inadequate to support derivation of oral RfD values for acute and chronic durations, and hence you conclude that derivation for acute and/or chronic derivations is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

I support the Environmental Protection Agency's (EPA) conclusion that the data are inadequate to derive an acute oral reference dose (RfD) for anatoxin-a. The majority of acute tests have exposed animals via i.p. administration and single dose oral testing has been at frank effect levels (FEL), which are not suitable to determine acute oral reference values. EPA was transparent and objective in its determination. In my opinion, the acute oral RfD is the most critical reference value for anatoxin-a due to the mode of action of this compound (neurological), rapid time-to-effect characteristics and the potential for high, single dose exposures in humans and animals. It would be preferable to conduct acute oral tests on canine and swine models, given their closer proximity to human physiology compared with rodents.

I support EPA's decision to not derive a chronic oral RfD for anatoxin-a, given the lack of research for this exposure duration and the additional uncertainty of using the principal subchronic studies (Astrachan & Archer 1981; Astrachan et al. 1980) to extrapolate to a chronic oral RfD. EPA's determination to not derive a chronic oral RfD was objective. EPA states in section 5.2.5.1 that one of the reasons for not developing a chronic oral RfD is that the limitations in the subchronic study preclude such an extrapolation. This is not very transparent, since concern for limitations in the Astrachan studies questions the suitability of this research as the basis for subchronic oral RfD derivation. EPA should be explicit in what limitations were used to justify not extrapolating subchronic → chronic reference values. In my opinion, the chronic oral RfD is less of a priority compared with research focused on characterizing an acute RfD for anatoxin-a.

2. The 28-day gavage study in mice (Fawell and James 1994; Fawell et al. 1999) was selected as the basis for the short-term oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

The selection of the Fawell and James (1994) and Fawell et al. (1999) 28-day gavage study in mice was an appropriate principal study to determine a short-term oral RfD. The Fawell studies are the most robust and suitable of all reported oral toxicity tests for anatoxin-a, including sufficient sample size, number of dose groups and examined treatment effects. A weakness of the 28-day gavage study is that the authors of the principal study (Fawell et al. 1999) determined that 0.1 mg/kg-day was the no-observed-adverse-effect-level (NOAEL), since two deaths could not be ruled out as treatment related. Their determination is inconsistent with the point-of-departure (POD) selected by EPA to derive a reference value for short-term oral exposure to anatoxin-a (see answer 3 below).

3. Is the designation of 2.5 mg/kg-day as the NOAEL for this study scientifically justified? Has the rationale for this designation been transparently and objectively described?

Yes, there is scientific justification to conclude that 2.5 mg/kg-day is an appropriate POD, despite a different conclusion from the study authors. Fawell et al. (1999) observed one death in the 0.1 mg/kg-day dose group and one death in the 2.5 mg/kg-day dose group (which constitutes 10% of the animals in a dose/gender group). The authors speculated and EPA concluded that these deaths were unlikely related to anatoxin-a exposure. EPA was transparent and objective in this determination citing a lack of a clear dose-response relationship, time-to-death, negative findings from the initial range-finding test and absence of treatment effects observed in surviving mice.

4. The 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al. 1980) was selected as the basis for the subchronic oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

I support EPA's conclusion that the Astrachan studies are appropriate as the basis for a subchronic oral RfD, despite the limited number of dose-groups (estimated ingestion rates of 0.05 & 0.5 mg/kg-day). Astrachan et al. (1980) examined several treatment level effects (although not all the results are reported in their paper) and utilized sufficient sample numbers. EPA was transparent and objective in its rationale.

5.Are the uncertainty factors applied to the points of departure (PODs) for the derivation of the short-term and subchronic RfD values scientifically justified and transparently and objectively described?

EPA has applied uncertainty factors (UF) to the points of departure (POD) that are scientifically justified and health protective, given the lack of information for oral exposure to anatoxin-a. The rationale for the UFs applied to the short-term and subchronic RfDs were transparent and objective. EPA applied 10X factors for interindividual susceptibility (which is especially justified given the two mortalities in the 28-day gavage study (Fawell et al. 1999) that could definitively ruled out as treatment related by the study authors). The full 10X UF for species differences and limitations in the database are justified as well by the lack of research on anatoxin-a. Research into the absorption, distribution, metabolism and excretion of anatoxin-a would reduce some uncertainty in characterizing this toxin. Additionally, oral exposure neurotoxicological tests in multiple species would be useful.

Cylindrospermopsin Toxicological Review

1.The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for acute, short-term or chronic exposure durations. Do you agree with this conclusion? Is the rationale for not developing acute or short-term oral RfDs transparent and objective? If you do not agree with the conclusion that the data are inadequate to support derivation of oral RfD values for acute and chronic durations, and hence you conclude that derivation for acute short-term and/or chronic derivations is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

I support EPA's conclusion that the available toxicity information is inadequate to derive an acute oral RfD for cylindrospermopsin given small dose groups at lethal concentrations (Seawright et al., 1999), i.p. administration near lethal levels (Falconer et al. 1999) and the observation that no purified toxin was used in acute testing. The Shaw et al. study (2000) appears to have observed a dose-severity response in the liver, however sufficient information on the experimental design was not reported. I support EPA's conclusion that the available toxicity information is inadequate to derive short-term and chronic oral RfDs since there is inadequate information or no information on these exposure durations, respectively.

2. The 11-week gavage study in mice (Humpage and Falconer, 2003) was selected as the basis for the subchronic oral RfD. Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

I support EPA's conclusion that Humpage and Falconer (2003) is the appropriate study for the subchronic oral RfD derivation of cylindrospermopsin. Humpage and Falconer (2003) conducted a well-designed study with multiple dose groups and examined several physiological, biochemical and histological endpoints. EPA was transparent and objective in selecting the Humpage and Falconer study, which was likely simplified by the fact that little toxicological research has been conducted on cylindrospermopsin.

3. The critical effect identified in Humpage and Falconer (2003) was increased relative kidney weight. Is selection of this finding as a critical effect scientifically justified? Is the rationale for selecting this effect transparent and objective?

Yes, the increased relative kidney weight identified in Humpage and Falconer (2003) is a scientifically justified critical effect for cylindrospermopsin. This critical effect is supported for several reasons. The kidney has been shown to be one of the primary targets of cylindrospermopsin (along with the liver) based on animal studies and on clinical findings in the Palm Island human poisoning incident (Blyth 1980; Griffiths and Saker, 2003). Additionally, the Humpage and Falconer study demonstrated a probable dose-severity response in the kidney (increased kidney weight at 60 µg/kg-day & decreased urine output at 120 µg/kg-day) that could ultimately progress to toxicity and in which statistically significant differences were detected between treated and control groups. EPA provided comprehensive support in section 4.5.2 for the relationship between the effects seen at 60 & 120 µg/kg-day. This section was transparent, and the references and supporting statements were presented objectively.

4. Benchmark dose (BMD) modeling was utilized to estimate a BMD and BMDL for increased mean relative kidney weight using 1 standard deviation above the control mean as the benchmark response level (BMR). After dropping the high dose group, the linear model adequately fit the data and produced an estimated BMD of 43.1 µg/kg-day and BMDL of 33.1 µg/kg-day. Was the modeling appropriately conducted and interpreted? The BMDL of 33.1 µg/kg-day was chosen as the POD for RfD derivation. Is the rationale for selecting this as the POD transparent and objective?

EPA selected appropriate models for fitting continuous data (i.e. kidney weight), provided graphic displays of plots to visually observe model fits and used Akaikie's Information Criterion (AIC) to assist with the selection among models. The choice of the linear model is supported since the AIC was relatively low and the line fit was high ($p = 0.98$) indicating that the model adequately described the data. Other models (power and polynomial) estimated the same BMDL, providing further confidence in this endpoint. Dropping the high dose in the Humpage and Falconer study is acceptable since the highest dose (240 µg/kg-day) was located far above the BMR and is a supported practice by EPA's Benchmark Dose Guidance (USEPA 2000). The choice of BMR = 1 standard deviation above the control mean is basically a default option for continuous data. EPA was objective in the choice of POD. To increase transparency, EPA should emphasize that the same reference value would be obtained using both BMD modeling and the traditional NOAEL approach in sections 5 and 6 of the toxicological review and provide more information on why the high dose group was eliminated (i.e. to optimize the modeling of data near the POD).

5. The conclusion was reached that the 11-week gavage study in mice (Humpage and Falconer, 2003) selected as the basis for the subchronic oral RfD study could not be utilized for the derivation of a chronic oral RfD due to the excessive uncertainty inherent in doing so. Do you agree with this conclusion? Is the rationale for not developing a chronic oral RfD transparent and objective? If you do not agree with the conclusion that the data are too uncertain to support derivation of oral RfD values for chronic durations, and hence you conclude that derivation for chronic derivations is appropriate, then please describe how you would recommend such derivation be completed and the rationale for such derivation.

I agree with the rationale for not deriving a chronic oral RfD for cylindrospermopsin based on the Humpage & Falconer (1999) study. While this study was well-conducted and may seem attractive for extrapolation of subchronic → chronic duration, a composite UF of 10000 is unacceptable. EPA was transparent and objective on this conclusion. It would result in a reference value of cylindrospermopsin that cannot be scientifically justified. Recently, a study was published in *Environmental Toxicology* on the chronic oral toxicity of cylindrospermopsin in mice (Sukenik et al. 2006) that was unavailable at the time this review was developed. Researchers exposed mice to cylindrospermopsin for 42 weeks, gradually increasing dose between 10 and 55 µg/kg-day. The authors determined that hematological effects were most pronounced and proposed a lowest-observed-adverse-effect-level (LOAEL) of 20 µg/kg-day. I suggest this paper as a topic of discussion in the Expert Peer Review meeting.

6. Are the uncertainty factors applied to the point of departure for the derivation of the subchronic oral RfD scientifically justified and transparently and objectively described?

A composite UF of 1000 (10 for interspecies, 10 for within human variability, 10 for database insufficiency) is warranted for cylindrospermopsin given the lack of research on this toxin. In my opinion, an acute oral toxicity test, preferably on canine or swine models, is warranted given the potential for single high dose exposure in recreational and drinking water scenarios. Additional research that would decrease the uncertainty in assessing cylindrospermopsin and deriving a reference value includes a multi-generational reproductive study and studies on absorption, distribution, metabolism and excretion.

Microcystins -RR, -YR and –LA Toxicological Review

1.The conclusion was reached that the available oral toxicity information was inadequate to support derivation of oral RfD values for microcystin-RR, -YR or LA. Do you agree with this conclusion? Is the rationale for not developing oral RfDs transparent and objective? If you do not agree with the conclusion that data are inadequate to support derivation of oral RfD values for microcystin-RR, -YR or LA, and hence you conclude that derivation is appropriate, then please describe how you would recommend such derivation(s) be completed and the rationale for such derivation(s).

I support EPA's overall conclusion that insufficient information and research exists to derive oral RfD values for microcystin -RR, -YR and -LA. As pointed out in the EPA toxicological review, the vast majority of research has been focused on MCLR. This is especially true for the –LA congener, which has no single high quality study of which I am aware. I support EPA's decision to not use i.p. studies for –YR and –RR as the basis for derivation of oral RfD values given the uncertainties in absorption, distribution and metabolism when administered via the i.p. route and extrapolated to the oral route. EPA has been transparent and objective in describing why oral RfDs cannot be derived using current information for these three congeners. I would recommend that EPA acknowledge that there are over 80+ known congeners of microcystins in Section 6 when discussing –RR, -YR and –LA, including a statement that a high degree of uncertainty exists regarding the toxicological properties of most congeners. Research that would increase our understanding of the toxicological properties of other congeners include 1) determining if a common mode of action exists among all or most known congeners, and 2) determining the potency of congeners relative to MCLR. The latter approach would be similar to the toxic equivalency factor (TEF) methodology for dioxins, furans and co-planar PCBs. In my opinion, this research should be based on oral toxicity studies and not i.p. administration, given the uncertainties in absorption, metabolism and distribution in translating i.p. exposure effects → oral exposure effects. While this research would be useful for mixture studies and risk assessment, it would be very expensive and resource intensive. Another approach to characterize the toxicity of multiple congeners would be to focus on mixture studies, similar to the research conducted by Falconer et al. (1994) on oral exposure of microcystin containing scum in pigs.

Microcystin LR Toxicological Review

1.The conclusion was reached that the available oral toxicity information was inadequate to support derivation of an acute oral RfD value. Do you agree with this conclusion? Is the rationale for not developing an acute oral RfD transparent and objective?

I support EPA's conclusion that no suitable studies exist to derive an acute oral RfD value. This conclusion was made for several reasons including inadequate reporting and examination of treatment endpoints (Fitzgeorge et al. 1994, Rogers et al. 2005), lack of untreated controls (Fawell et al. 1999, Yoshida et al. 1997) and inadequate dosing groups (Ito et al. 1997). EPA was transparent and objective in the determination that no studies are currently available that are sufficient to derive an acute oral RfD. Similar to anatoxin-a and cylindrospermopsin, research focused on the derivation of an acute oral RfD for MCLR is warranted, given the potential for acute exposure in drinking water or recreational scenarios. Canine or swine models should be considered over rodents, given their closer proximity to human physiology.

2.The 28-day drinking water study in rats (Heinz, 1999) was selected as the basis for the short-term and subchronic oral RfDs. Is the selection of this study as the principle study appropriate? Is the rationale for selecting this study transparent and objective?

The selection of the Heinze study is appropriate as the basis for a short-term oral RfD and EPA was transparent and objective in this determination. Heinze provided adequate data reporting and established a dose-response relationship for several endpoints. For the subchronic oral RfD, EPA also selected Heinze (1999) over (Fawell et al. 1999). From what I can determine, this decision was based on a lower POD in the Heinze study compared with the Fawell study. However, I think the Fawell studies are more robust overall. The Fawell study lasted 90 days, which is approximately 3-fold longer than the Heinze study. Fawell et al. tested more dose groups and used a larger sample size than the Heinze study, identifying a clear NOAEL and LOAEL. In contrast, the Heinze study did not identify a NOAEL and did not adjust dosage for unconsumed drinking water. I recognize EPA's dilemma that selecting Heinze et al. as the basis for a short-term reference value and Fawell et al. as the basis for a subchronic reference value would yield nonsensical RfD values (i.e. a lower reference value for short-term exposure compared to subchronic exposure) and suggest this issue for discussion at the Expert Panel meeting. EPA should be more explicit on the rationale for choosing Heinze for the subchronic reference value. Was it based solely on providing a lower POD compared to Fawell et al (1999)? Was consistency between subchronic and short-term reference values a factor in study selection? Were other factors relevant?

3. Liver toxicity observed by Heinze (1999) included liver lesions, serum enzyme changes and changes in relative liver weight. All three of these endpoints were considered for determining the point of departure for RfD derivation. BMD modeling was utilized to estimate a BMD and BMDL for each of these endpoints. Was the modeling appropriately conducted and transparently and objectively presented?

EPA was thorough and transparent with the BMD modeling on liver lesions, enzyme changes and relative liver weight. All three endpoints demonstrated a dose-response trend and were appropriately examined to determine which response was appropriate for a POD.

For the purposes of BMD modeling, the moderate and severe liver lesions categories reported by Heinze (1999) were combined and the BMDs and BMDLs for these lesions estimated by the log probit model were 11.0 and 6.4 µg/kg-day, respectively. The BMDL of 6.4 µg/kg-day was chosen as the POD for RfD derivation. Is the rationale for selecting this as the POD transparent and objective? Is selection of liver lesions as a critical effect scientifically justified? Is the combining of moderate and severe lesions scientifically justified? Is the rationale for selecting this effect transparent and objective?

EPA was objective and transparent in selecting liver lesions as the POD for RfD derivation. To derive this POD, EPA grouped moderate and severe lesions, which is an accepted practice for categorical data. However, four slight liver lesions were observed in the low dose group. It would be more transparent if EPA grouped the categorical lesions (moderate + severe) as "lesions > slight severity" in the BMD model. The rationale for selecting liver lesions as the critical effect is scientifically justified, consistent with other studies, and provided the lowest POD. Furthermore, liver lesions are likely to be irreversible and potentially severe.

4. Data from the 90-day gavage study in mice conducted by Fawell et al. (1999) was considered for the derivation of the subchronic RfD. The BMDL from the Heinze (1999) study (6 µg/kg-day) is approximately an order of magnitude lower than any of the BMDL values derived from endpoints from the 90-day gavage study (57-66 µg/kg-day) therefore, the BMDL of 6 µg/kg-day from Heinze (1999) was chosen as the POD for subchronic RfD derivation. Do you agree with this decision? Is the rationale for selection 6 µg/kg-day as the POD transparent and objective?

EPA provides sufficient rationale for using 6 µg/kg-day as the POD. However, as described in question 2 above, I think the Fawell et al. (1999) study had a more robust design for subchronic exposure. EPA should be more transparent about the decision to use Heinze as the principal study over the Fawell et al. study.

5. The 18-month study drinking water study in mice (Ueno et al. 1999) was selected as the basis for the chronic oral RfD. This study used only a single dose level and identified a freestanding NOAEL but was chosen for RfD derivation because it was a well-conducted study of chronic duration and employed a relevant exposure route (drinking water). Is the selection of this study as the principal study appropriate? Is the rationale for selecting this study transparent and objective?

The choice of the Ueno et al. (1999) study was appropriate as the principle study to derive a chronic oral RfD. The Ueno study evaluated 100 control and 100 treatment female mice. While no male mice were evaluated, it is unlikely that male-specific effects would have been noted, given the ultimate findings of this study. Ueno et al. evaluated several toxicological, physiological and pathological endpoints. The observation of a few statistical differences between control and treated groups were not considered to be toxicological significant, which appears to be supported by the lack of treatment-related effects of biologic plausibility with other studies of MCLR. EPA was transparent and objective in the choice of this study as the basis for the derivation of a chronic oral RfD, citing the quality of this study and large sample size.

6. Are the uncertainty factors applied to the points of departure for the derivation of short-term, subchronic and chronic oral RfDs scientifically justified and transparently and objectively described?

Short-term & subchronic oral RfD: I suggest that an uncertainty factor (UF) of 300 be applied to the POD for these two exposure durations. The basis for the 300X UF is: 10 for interspecies differences, 10 for interindividual variability and 3 for insufficient database. The justification for 3 instead of 10 for insufficient database reflects the detailed research that has been conducted on the mode of action for MCLR in liver toxicity (thus lessening the weight of a neurotoxicity test in judgment, especially at non-acute exposure durations), observation of similar responses in the liver among multiple species (including humans, swine, mice, rats) and the relatively high NOAEL (compared to the Heinze BMR for liver lesions) for maternal and developmental effects reported by Fawell et al. (1999).

Chronic oral RfD: For chronic oral exposures, an UF = 1000 is appropriate and scientifically justified. EPA was transparent and objective in its application of the UFs for interspecies differences,

interindividual differences and insufficient database (especially considering that the tumor promoting ability of MCLR is uncertain).

Application of the UFs suggested above would yield oral reference values of:
Short-term oral RfD = 0.02 µg/kg-day
Subchronic oral RfD = 0.02 µg/kg-day
Chronic oral RfD = 0.003 µg/kg-day

Cancer Assessments for Anatoxin-a, Cylindrospermopsin and Microcystins (LR, RR, YR and LA).

1.Do the available data support the conclusion that the database for each of these toxins provides inadequate information to assess carcinogenic potential based on the weight-of-evidence categories in the EPA 2005 *Guidelines for Carcinogenic Risk Assessment*? Please describe the basis for your view.

Anatoxin-a: Clearly, the information on anatoxin-a is insufficient to assess carcinogenic potential as no research has been conducted to date. I support EPA's conclusion that there is *inadequate information to assess carcinogenic potential*.

Cylindrospermopsin: Very little research has been conducted on the carcinogenic potential of cylindrospermopsin. Falconer and Humpage (2001) assessed the tumor promoting ability of cell extracts in mice. Neoplastic changes were observed in 5 of the treated mice and none in the controls. This difference was not statistically significant, and the pattern of tumors was not clear, including the observation of widely different cancers, that provided inconsistent endpoints of carcinogenic potential. Based on these factors, I support EPA's conclusion that there is *inadequate information to assess carcinogenic potential* of cylindrospermopsin.

Microcystin LR: MCLR is suspected of tumor promotion activity (Ohta et al. 1994, Falconer & Buckley 1989, Falconer 1991, Nishiwaki-Matsushima et al. 1992) and mechanistic studies have demonstrated the ability of MCLR to cause cell proliferation at low doses (Hu et al. 2002) or upregulation of transcriptional factors in cell proliferation genes (Zhao and Zhu 2003). Several epidemiological investigations have examined the association between drinking from cyanobacteria ponds/ditches in China and prevalence of liver or color cancer, with conflicting results (Ueno et al. 1996, Yu et al. 1989, Yu et al. 2002, Chen et al. 2002). I acknowledge that these investigations have large confounders, including a limited ecological study design, potential for aflatoxin exposure and a high incidence of hepatitis infection among the studied populations. However, some of the associations are suggestive of a role for MCLR in the development of cancer and studies on the tumor promoting ability of MCLR are compelling. At this time, I recommend that MCLR be categorized as *suggestive evidence of carcinogenic potential.* A cancer bioassay conducted by the National Toxicology Program would be very beneficial to further understand the carcinogenic potential of MCLR.

Microcystin RR, -YR and –LA: I support EPA's conclusion that MCRR, -YR and –LA be categorized as *inadequate information to assess carcinogenic potential*.

Christopher Williams

EPA Toxicological Reviews of Cyanobacterial Toxins

General Questions
Charges:

Are the toxicological reviews logical, clear, and concise? Has EPA accurately, transparently and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?

Yes, I believe the EPA has performed an admirable job in developing a reasonably comprehensive compilation of related and appropriate studies to assess oral RfD values for the various exposure durations. In most instances, the studies presented are well defined, summaries are concisely described, and the positives and negative aspects of the studies are clearly stated and identified.

Are you aware of additional studies that should be considered in the assessment of the noncancer and cancer health effects of these toxins?

On occasion, but infrequently, I have stated in the appropriate sections that other studies are available for consideration. In general, however, I have found that the compilation has been complete with my current knowledge and experience. I am not nor do I claim to be an expert on the most current research completed. There is a new epidemiological study, performed in August of 2006, that is being finalized by the Dr. Lorraine Backer at the CDC for recreational exposure to microcystins, but I do not believe this would assist with the current RfD assessment/determinations. Microcystins were present in low concentrations (2-6 ug/L) in lake water, microcystins were observed in filtered air samples indicating the potential of inhalation exposure, and blood was drawn from human participants for microcystin analysis (analysis still ongoing; Dr. W. Carmichael and WSU).
Furthermore, there is a substantial amount of non-English literature and research that can be applied to the derivation and determination of these RfD estimates. Depending on the objective of this assessment, the time should be taken to review these studies and evaluate if they would/could significantly assist. The lack of this information may significantly affect the overall completeness and perception of this review.

3) Please discuss research that you think would be likely to reduce uncertainties in the reference values for future assessments?

Again, in the appropriate sections, I have indicated my ideas as to what areas of research I feel need to be prioritized. In general, acute and short-term exposure studies for all of the cyanotoxins in question should be prioritized as these types of exposures are the most relevant to human health yet still lack vital information. The compounds in question are potent toxins that can and will cause human health problems, including mortality. A lack of purified reference materials for the cyanotoxins (except maybe MLR) strongly inhibits this process and the production of these compounds needs to be supported and expanded. If and when purified toxin is available, studies with a greater number of treatment levels need to be designed and the reduction/elimination of free-standing NOAELs with true NOAELs needs to be determined. Obviously, the use of BMD analysis would be best for the determination of RfD-values. Furthermore, reproduction of principle studies and verification of their results should also be performed. Sub-chronic and chronic exposure studies should be developed for cylindrospermopsin and the microcystins as research has indicated that these compounds do have the potential to interfere with cellular processes involved with the cell cycle and DNA. Again, a lack of purified compounds and a lack of available mass will significantly inhibit such research as greater mass will be necessary for longer

duration exposures. Furthermore, epidemiological studies and surveillance studies need to be designed and implemented to identify exposure potentials and susceptible/impacted populations.

Anatoxin-a

Charges:

Do you agree with the finding that the available oral toxicity data for anatoxin-a precludes the determination of RfD-values for acute and chronic exposures?

Acute RfD values: Yes, I believe the data presented and that I am knowledgeable of are insufficient for the determination of a specific RfD for acute exposure to anatoxin-a. The rationale of no well-documented human exposure cases and/or associated dose-response relationships is clearly described. The lack of sensitive endpoints other than mortality may be simplistic for a fast acting toxin and, at the present time, technologically and analytically very difficult to provide. It should be stated, however, that anatoxin-a might contain the greatest capacity and provide the greatest risk to elicit acute human toxicosis due to its mode and rapidity of action. Our laboratory has observed/reported environmental anatoxin-a levels as high as 156 ug/L as well as the presence of this compound in drinking water samples on several occasions. The highest concentration of anatoxin-a in drinking water was 8 ug/L. More research effort really needs to placed and emphasized in this subject area as short exposures to relatively high environmental concentrations can have significant and rapid health implications. I feel that acute exposure to anatoxin-a, potentially, may be the greatest human health concern of any of the cyanotoxins.

Chronic RfD values: Yes, I believe the data presented are insufficient for the determination of a specific RfD for the estimation of chronic exposure to anatoxin-a. The rationale that the sub-chronic oral data could not be used to extrapolate a chronic RfD across exposure durations is clear and justifiable. In general, anatoxin-a may not be a high risk compound for long-term consistent exposure due to its high water solubility (easily depurated), a half-life of approximately 14 days under normal environmental conditions (Smith and Sutton, 1993) and an increase in breakdown under high pH conditions (normal for bloom events). Furthermore, the long-term production of anatoxin-a by cyanobacteria has not been reported in either water or feed supplements.

Is the selection of the Fawell and James (1994) and Fawell et al. (1999) appropriate to be the principal study for determining the short-term oral RfD?

Yes, I believe that the Fawell and James (1994) in conjunction with Fawell et al. (1999) are the most appropriate studies performed at present to best estimate oral RfD for short-term exposures. These studies are well designed and investigate the effects of anatoxin-a over a 5-28 day period, a period of time consistent with short-term exposures (7 days) and the development of a shot-term oral RfD (30 days). Although the five-day study did not include an appropriate control group and the number of exposed mice were relatively low (n=4), the preliminary results did exhibit a dose response effect, neurotoxic responses were observed and this study did provide preliminary evidence that 2.5 mg/kg-day might be a close approximation for a short-term NOAEL. In the principle study (28-day exposure), negative controls were used, replication between treatments was high (n=20), and multiple relevant endpoints were evaluated and analyzed. Administration was daily of a commercial product via gavage so exposure was consistent and extraneous toxic compounds were not present. One major problem with this study is that a purity of the anatoxin-hydrochloride was not provided and actual concentration levels were not confirmed/verified by LC/MS. For comparison sake, the oral LD_{50} for anatoxin-a is $\geq$ 5.0 mg/kg bw in mice (Fitzgeorge et al. 1994).

In the studies by Fawell and James (1994) and Fawell et al. (1999), EPA adjusted the NOAEL to 2.5 mg/kg-day, is this scientifically justified? Is this rationale transparent and objective?

Mortality was observed in this study at both 0.5 and 2.5 mg/kg-day. If one makes the assumptions that there is no difference between sexes in their response to short-term exposures to anatoxin-a and aggregates the data than a consistent 5% mortality rate was observed and may be considered an effect. It is true that if we do aggregate the data than a dose response was still not observed between treatment levels. This lack of a dose response for mortality, at what was considered tolerable daily levels, might be a reasonable result for a sub-lethal level dose. Increases in certain relevant indices

3-cont.) at the cellular level should be expected, if one selects the correct indices to evaluate. An increase in mean hemoglobin content might be a relevant (short-term) response to complications in respiration as might increased sodium levels if sodium channels are maintained in an open position due to the presence of anatoxin-a. This 5% rate is very similar to that rate (10%) reported by Australian researchers (SAWater, M. Burch) for individual sensitivity in humans to cyanotoxins in general. If approximately 5-10% of the population are overly sensitive to cyanotoxins than significant changes in secondary indices in non-sensitive individuals may not be observable or relevant to individuals who exhibit hypersensitivity. Unfortunately, it appears that this study was only performed once and therefore reproduction of these results was not confirmed and cannot be validated.
On the other hand, corroboration of both the 5-day study (FEL = 6.2 mg/kg-day) and the developmental toxicity studies (free standing 2.5 mg/kg-day) producing similar results to that of the 2.5 mg/kg-day value during the 28-day exposure does suggest that this value is consistently obtainable. Furthermore, the lack of a neurotoxic response (rapidity and behavior) in conjunction with mortality suggests that use of this value as a RfD might be reasonable.
In my experience, however, FELs can be significantly different than NOAELs and the justification of anatoxin-a as a developmental toxin is unclear and therefore a freestanding NOAEL may be irrelevant. In my opinion, the use of 2.5 mg/kg-day as a RfD is very questionable and needs more conclusive information. Furthermore, the close proximity of the modified NOAEL to the oral LD_{50} for anatoxin-a ($\geq$ 5.0 mg/kg bw in mice, Fitzgeorge et al. 1994) gives me concern. To be conservative and truly provide a safe concentration dosage I would suggest using 0.1 mg/kg-day as the POD.

Is the selection of Astrachan and Archer (1981) and Astrachan et al. (1980) as the principle studies for estimating a sub-chronic oral RfD appropriate? Is the rationale transparent and objective?

Yes, I believe that the Astrachan publications, although relatively old in nature (1980, 81), are the most relevant and appropriate studies for the determination/estimation of an RfD for sub-chronic exposure to anatoxin-a. Anatoxin-a, however, is a fast acting neurotoxic compound whose mode of action is not conducive to long-term complications, there has been no reported genotoxic potential and therefore there are not a lot of studies that have investigated potential long-term exposure effects. The advantages of these studies are that replication per treatment is very good (n=20) and the amount of endpoints analyzed during and after the study was extensive. A few negative aspects of the studies are that treatment levels consisted of only two exposure concentrations, which makes the determination of a highly accurate RfD impossible. Furthermore, the quantification of the actual concentration levels ingested may be questionable due to very little information on purity, actual volume ingested during the study, and analysis of the concentration of the toxin source over time. Because of the uncertainties in this study, I believe further investigations need to be performed to verify and confirm the NOAELs from this study.

Are the uncertainty factors applied to the points of departure for the derivation of the short-term and sub-chronic RfD values scientifically justified, transparent, and objective?

Yes, I believe the uncertainty factors applied to the points of departure are consistent with those recognized by general toxicology guidelines. Papers by Clarkson (Principles of Risk Assessment, 1992) and Purchase (Risk Assessment: Principles and Consequences, 2000) describe similar types of uncertainty factors for interspecies and intraspecies extrapolations. I believe the basis for utilization of these safety factors is clear, scientifically justifiable and objective.

Notes: I believe that Fitzgeorge et al (1994) did report on the intranasal exposure of mice to anatoxin-a.

Cylindrospermopsin

Charges:

Do you agree that the available oral toxicity data was inadequate to support derivation of an oral RfD values for acute and short-term exposure durations? Is the rationale for this conclusion transparent and objective?

Yes, I would have to agree that the available oral toxicity data for NOAEL values using purified cylindrospermopsin are inadequate to support a derivation of an oral RfD value for both acute and short-term exposure durations. Several studies exist that describe potential oral toxicity but are relegated to aqueous cell extracts (that may have additional toxic compounds present) or report LD_{50} values for other exposure routes other than oral intake. Similar to anatoxin-a, more research needs to be directed to this subject area because acute toxicity and short-term exposures are the most likely exposure durations for both environmental and drinking water contamination. Our laboratory has observed environmental concentrations as high as 220 ug/L and 98 ug/L in post-treatment drinking water samples.

Is the selection of the study by Humpage and Falconer (2003) for the derivation of sub-chronic oral RfD appropriate, transparent, and objective?

Yes, I believe the study performed by Humpage and Falconer (2003) is the most appropriate for the derivation of a sub-chronic oral RfD. Purified cylindrospermopsin was used as the chemical agent, administration via gavage was used as the route of exposure, replication between treatment levels was reasonable (n= 6-10), quantifiable behavioral data was reported, clinical examination performed, histopathology of internal tissues characterized, as well as a multitude of biochemical indices analyzed. Exposure duration was appropriate for sub-chronic testing at 11-weeks.

Is the selection of relative kidney weight (Humpage and Falconer 2003) as a critical effect scientifically justified? Is the rationale for selecting this effect by the EPA transparent and justified?

An increase in relative kidney weight does not by itself indicate an adverse effect and is more of a general systemic indicator of abnormal metabolism that cannot identify a specific cause and effect relationship. However, an increase in relative kidney weight that is: 1) positively correlated with dose concentration, 2) associated with proximal renal tubular damage, 3) reported in conjunction with a decrease in urine protein excretion, and 4) known to be a target tissue of cylindrospermopsin justifies the conclusion that this is a critical effect caused by sub-chronic exposure. Whether or not hyperplasia of renal cells is the true cause for this increase in weight is speculative, at best, but never the less is justifiable as a reasonable endpoint of concern worthy of further research interests.

In the study performed by Humpage and Falconer 2003, benchmark dose modeling was used, was the modeling appropriately conducted and interpreted? Is the rationale for selecting the BMDL of 33.1 ug/kg-day by the EPA transparent and objective?

My experience with BMDL modeling is limited, from what information I have obtained it does appear that the modeling was performed and interpreted correctly. Homogeneity of variance analysis was performed across treatment levels and was satisfied. Several different models were tested (Hill, linear, polynomial, and power models) and compared to determine which model provided the best fit for the data being used. Akaike's Information Criteria was used and evaluated to determine the best model. A BMDR of 43.1 ug/kg-day was estimated after exclusion of the high dose category and fitting the data to a linear model. **However, after the elimination of the high dose treatment it appears to me that the goodness of fit is significantly reduced (p-values = 0.84-0.98 usually p < 0.10) and that the BMDL of 76.6 ug/L (p=0.01) may need to be used as the POD (this may be an entry error).** If I am misreading the tables and graphs than the close approximation of the NOAEL for relative renal weight increase (30 ug/kg-day) to that of the BMDL (33.1 ug/kg-day) does suggest that this value might be a good approximation as a point of departure and therefore a reasonable estimate for the RfD for sub-chronic

4-cont.) exposures. I would agree that the use of 33.1 ug/kg-day as a more conservative estimate is a safe and responsible practice.

5) The EPA determined that the Humpage and Falconer 2003 study could not be used to derive a chronic oral RfD due to excessive uncertainty, do you agree with this conclusion? Is the rationale transparent and objective?

Extrapolation of data from sub-chronic exposure to chronic exposure usually deserves an uncertainty factor of 10. The general life expectancy of albino mice can be estimated at approximately 700 days (Menahan and Kemp, Mech. Ageing Dev, 1982). Sub-chronic exposures are usually 10% of the total life span of a given individual and chronic exposures are defined as being a significant fraction of the animal's lifetime. The Humpage and Falconer (2003) study would incorporate approximately 10% (77 days divided by 700) of the lifetime of mice. Therefore it would appear that a UF of 10 for extrapolation from sub-chronic to chronic exposure is warranted. If 1000-fold uncertainty is unacceptable to the EPA then derivation of a chronic exposure RfD is certainly not supported. Under EPA suggested guidelines for risk assessment an UF of no greater than 10,000 is recommended to be used for derivation calculations and therefore I believe the Humpage and Falconer study could be used as a first estimate for chronic exposure.

Are the uncertainty factors applied to the point of departure for the derivation of the sub-chronic oral RfD scientifically justified, transparent, and objective?

Similar to my comments for anatoxin-a, the uncertainty factors applied by the EPA to the point of departure for estimating the RfD for sub-chronic exposure is consistent with other previous risk assessments for other potentially hazardous compounds and is consistent with the accepted principles of risk assessment. The reasons for using these uncertainty factors is clear and objective.

Microcystins (LR, RR, YR and LA)

Microcystin-LR

Charges:

The conclusion was reached that the available oral toxicity information was inadequate to support derivation of an acute oral RfD value, do you agree with this finding? Was EPA's rationale transparent and objective?

Although several studies have investigated acute toxicity exposure with appropriate animal models to the oral administration of purified MLR, not one of these studies was able or was designed to identify a NOAEL or provide a satisfactory data set for BDML analysis. For this reason it is justifiable to indicate that inadequate data is available for the determination of a specific RfD for acute oral toxicity. Our laboratory has consistently observed environmental concentration levels of total microcystins between 1000-8000 ug/L during bloom events, has observed on a number of occasions microcystins present (quantifiable) in drinking water samples, and concentrations in post-treated water samples greater than 1 ug/L (max. = approx.10 ug/L.) and therefore such a determination is necessary.
Data was provided that showed an FEL of 1580 ug/kg and an oral LD_{50} of 5,000 ug/kg as identified by Fawell et al. (1999), an FEL of 12.5 mg/kg (12,500 ug/kg) identified by Yoshida et al. (1997), and the closest approximation to a NOAEL was the free-standing LOAEL of 500 ug/kg identified by Ito (1997). If the 500 ug/kg LOAEL is used and an uncertainty factor of 10,000 (an additional UF = 10 for extrapolating from LOAEL to NOAEL) is applied than the acute oral RfD would be approximately 0.05 ug/kg.

The study by Heinze (1999) was selected as the principle study for deriving a value for the short-term and sub-chronic oral RfDs, is the selection of this study appropriate and is the rationale transparent and objective?

2-cont.) Yes, I believe the study by Heinze (1999) is an appropriate principle study in which to base the determination of the short-term oral RfD value for microcystin-LR. In this study, purified MLR was used as a toxin source (although the concentration was not adequately confirmed), an appropriate/relevant mammalian animal model was used (rat), two treatment levels plus a negative control were employed, replication was strong (n=10), multiple endpoints were examined, and a dose response relationship was observed that clearly identified a specific harmful effect (liver lesions) in the main tissue of concern for MLR exposure.

The use of Heinze (1999) for the determination of a sub-chronic RfD seems less suitable. Although the same positive aspects of this study still holds true the duration of this study (28-days) does not approximate that typically used for sub-chronic duration studies (10% of life expectancy). The presence of Fawell et al. (1999), which investigated the oral exposure of mice to purified MLR for 90-days, seems more justifiable and reasonable. The explanation of why the authors decided to defer to the Heinze study was clear. The difference between the NOAEL and the LOAEL between these two studies is minor and defaulting to the more conservative value may be appropriate. In the Heinze study, however, as previously indicated, the toxin source was obtained from a commercial vendor and the actual concentration levels of MLR used were not reanalyzed and confirmed. It is understood by most researchers who purchase commercial cyanotoxin standards that such items can, at times, contain significantly different concentrations than labeled. This possibility in conjunction with the limited duration of exposure suggests to me that the more appropriate study was the Fawell et al. study (1999). The use of data from the Fawell study indicated a tolerable daily intake of 0.04 ug/kg-day
Of course, defaulting to a lower RfD value when there might be some questions concerning interpretation is a safe and acceptable practice. Furthermore, the chronic study performed by Ueno et al. (1999: POD = 3 ug/kg-day) further substantiates the use of the lower POD value of 6 ug/kg-day (an RfD of 0.006 ug/kg-day).

In Heinze (1999), BMD modeling was utilized to estimate a BMD and a BMDL, was the modeling appropriately conducted and the rationale transparent and objective? Was the rationale for selecting the point of departure (6.4 ug/kg-day) transparent and objective? Is the selection of liver lesions scientifically justified? Is the rationale for selecting this effect transparent and objective?

Yes, it does appear that the modeling was performed correctly and appropriately. Numerous different models were utilized to determine which model provided the best goodness of fit as well as the lowest

Akaike Information Criteria value. Several different data sets were modeled that demonstrated a dose response relationship and the lowest of these values (the most conservative estimate) was used to develop the short-term RfD. However, it is not entirely clear to me as to why 6.4 ug/kg-day was accepted as the point of departure. From the data reported in Table 5-3, there appears to be little difference between X^2 values, goodness of fit p-values, and AIC values between the different models examined. If this is truly the case (no statistics are given), than I would think the more conservative estimates of either 1.2 (less degrees of freedom and lower AIC) or 3.9 ug/kg-day would be more appropriate. The use of these values would significantly lower the RfD for oral short-term exposure. I understand the lower degrees of freedom in the log-logistic model may be restrictive and provides for a greater AIC, while the goodness of fit and the AIC for the remaining appropriate models seem comparable. If this is the case, then I would suggest that the more conservative BMDL of 3.92 ug/kg-day be used as the POD.

Do you agree with the decision to select the BMDL of 6 ug/kg-day as the point of departure for sub-chronic RfD derivation? Is the rationale transparent and objective?

If the POD from Heinze (1999) is the accepted study because it provides the safer more conservative estimate, than my questions to charge 3 of this section are still appropriate, i.e., shouldn't 1.2 or 3.9 ug/kg-day be the PODs for the determination of the RfD for sub-chronic oral exposure? See comments to charge 3.

Is the selection of Ueno et al. (1999) as the principle study for the determination of a chronic oral RfD appropriate? Is the rationale for this selection transparent and objective?

Yes, the Ueno et al. (1999) study was appropriately designed to observe the chronic effects (18 months) of purified MLR in drinking water on mice. Although the toxin source was a pre-concentrated algal bloom water sample, it was characterized as 95% pure by HPLC which confirms composition and concentration levels. The overall design was very good. Replication was strong (usually n=10), multiple endpoints at the individual, tissue, and biochemical levels were examined, and multiple time points were evaluated for biochemistry and histopathology between treatment and control groups. One negative aspect of this study was that only one treatment group (20 ug/L) was used to compare against negative controls and no differences between these two groups were considered to be "toxicologically significant". A significant increase in cholesterol was observed at the end of the study at month 18 in treated mice but no specific toxicological significance with regard to exposure to MLR was given this effect. Because of this fact, only a freestanding NOAEL of 3 ug/kg-day could be identified.
The Ueno et al. study is more appropriate than either of the Ito et al. (1997), due to lack of purity of toxin source, lower replication, fewer endpoints examined (only liver, no biochemistry), and a lower duration of exposure (28 weeks vs. 18 months). The Ueno et al. study (1999) was more appropriate than the Thiel study (1994) because this study used MLA and not MLR and was not a specific peer reviewed publication describing the particular methods and analyses but a conference summary of this investigation.

Our laboratory has observed quantifiable microcystin levels (< 1ug/L) in several Florida lakes to be a year-round phenomenon therefore constant exposure to environmental microcystins is feasible. This would also indicate that the potential exists for microcystins to be present in raw surface waters used for drinking water production on year-round basis as well.

Are the uncertainty factors that were applied to the points of departure for the derivation of the short-term, sub-chronic and chronic oral RfDs scientifically justified, transparent, and objectively described?

Similar to my comments for anatoxin-a and cylindrospermopsins, the uncertainty factors applied by the EPA to the point of departure for estimating the RfD for short-term, sub-chronic and chronic exposures are consistent with other previous risk assessments for other potentially hazardous compounds and is

consistent with the accepted principles of risk assessment. The reasons for using these uncertainty factors are clear and objective.

The conclusion was reached that the available toxicity information was inadequate to support derivation of oral RfDs values for microcystin-RR, -YR or –LA. Do you agree with this conclusion? Is the rationale for not developing oral RfDs transparent and objective? If you do not agree, please describe how you would recommend such derivations be completed and the rationale for such derivations.

Yes, I would agree that little information exists as to the oral toxicity of microcystin-RR, -YR and -LA . More research effort needs to be directed to these secondary but commonly observed microcystin congeners and their potential impact on human health. First of all, however, before such an effort can be initiated, more attention needs to be placed into the purification of these compounds. In general, a lack of supply for purified cyanotoxin standards, at documented and verifiable concentrations, exists and severely limits the ability for this type of work to be performed. Without reference standards, exposure studies cannot be designed properly and performed to determine RfD values and analysis is limited to those who have small inventories. Again acute toxicity and short-term exposure should be emphasized if and when standards are available. Monitoring of microcystin producing bloom events needs to characterize the concentration levels and frequency of occurrence for both recreational and drinking water exposure. Microcystin congeners can have significantly different toxicities, chemical properties, such as hydrophilicity and hydrophobicity, and potential tissue and cellular distribution and metabolism than that of MLR. Until such work can be performed and validated than a conservative effort may be to use the estimated RfD values for MLR, as it is commonly accepted that MLR has the greatest toxicity of the microcystin compounds reported to date.

Cancer Assessment for Anatoxin-a, Cylindrospermopsin and Selected Microcystins (LR, RR, YR and LA)

Charges:

Do the available data support the conclusion that the database for each of these toxins provides inadequate information to assess carcinogenic potential based on the weight-of-evidence categories in the EPA 2005 Guidelines for Carcinogen Risk Assessment? Please describe the basis for your review.

Yes, I would agree that under the EPA 2005 Guidelines for Carcinogenic Risk Assessment insufficient data (primarily a lack of dose response data using purified compounds that indicate the formation of carcinogenic characteristics) exists to assess carcinogenic potential for anatoxin-a, cylindrospermopsin, and the selected microcystins. However, it should be pointed out that studies investigating microcystin-LR (and therefore probably the -RR, -YR, and -LA) and cylindrospermopsin have provided adequate data (interference in vital cell cycle processes and DNA/RNA structure) to indicate that these compounds need further evaluation as potential tumor-promoting compounds. Epidemiological studies are just now being designed and implemented by the CDC and may lead to longer term evaluations that may be able to more closely track and identify these types of relationships. Obviously, studies need to be designed to specifically address these questions and populations with greater risks identified. In Florida, it is not uncommon for lakes to exhibit year-round toxin producing blooms for multiple years, for yards to be irrigated with bloom containing water, for well (drinking) water to be under the influence (leaching) of bloom containing lakes, for surface waters to be used as a source for providing bottled water and for toxin containing water to be used as a source for producing public potable water supplies. Also, more and more nutritional companies are developing products that contain high concentrations of (potentially toxin containing) blue-green algae.